The effects of organic environmental toxicants on hard tissue formation in developing tooth

An *in vitro* study in mice

Eija Salmela

Institute of Dentistry

Department of Pediatric and Preventive Dentistry

Faculty of Medicine

University of Helsinki

Helsinki University Biomedical Dissertations No. 152

Academic Dissertation

To be presented for public examination, with the permission of the Faculty of Medicine, University of Helsinki, in the main auditorium of the Institute of Dentistry on May 27th 2011, at 12 noon

Supervised by:

Professor Satu Alaluusua, DDS, PhD
Department of Pediatric and Preventive Dentistry
Institute of Dentistry
University of Helsinki, Finland

Docent Pirjo-Liisa Lukinmaa, DDS, PhD
Department of Oral Pathology
Institute of Dentistry
University of Helsinki, Finland

Reviewed by:

Professor Irma Thesleff, DDS, PhD
Institute of Biotechnology
University of Helsinki, Finland

Docent Matti Viluksela, PhD
Department of Environmental Health
National Institute for Health and Welfare
Kuopio, Finland

Opponent:

Professor Leo Tjäderhane, DDS, PhD
Institute of Dentistry
University of Oulu, Finland

ISBN 978-952-10-6961-1 (paperback)
ISBN 978-952-10-6962-8 (PDF)
ISSN 1457-8433
Unigrafia Oy Yliopistopaino
Helsinki 2011

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

ABBREVIATIONS

ABSTRACT

1. REVIEW OF THE LITERATURE 1
   1.1. Tooth development 1
      1.1.1. Initiation and morphogenesis 1
      1.1.2. Differentiation of the dental cells 2
      1.1.3. Formation and mineralization of the dental hard tissues 4
         1.1.3.1. Predentin and mineralized dentin 4
         1.1.3.2. Enamel 8
         1.1.3.3. Root cementum 10
   1.2. Disturbed dental hard tissue formation 11
      1.2.1. Background and clinical aspects 11
      1.2.2. Inherited defects 12
         1.2.2.1. Dentin defects 12
         1.2.2.2. Enamel defects 12
      1.2.3. Defects caused by systemic environmental factors 13
   1.3. Environmental toxicants 13
      1.3.1. Dioxins and dioxin-like compounds 13
         1.3.1.1. Nature and human exposure 13
         1.3.1.2. Mechanisms of action 14
         1.3.1.3. General effects 14
         1.3.1.4. Effects on tooth development 15
      1.3.2. Non-halogenated polycyclic aromatic hydrocarbons 16
         1.3.2.1. Nature, human exposure, general effects and mechanism of action 16
         1.3.2.2. Effects on tooth development 17
      1.3.3. Organic tin compounds 18
         1.3.3.1. Nature, human exposure and general effects 18
         1.3.3.2. Effects on tooth development 19
   1.4. Fluoride 19
      1.4.1. Sources and toxicity 19
      1.4.2. Effects on tooth development 20

2. AIMS OF THE STUDY 22

3. MATERIALS AND METHODS 23
   3.1. Teeth and organ culture 23
   3.2. Preparation of explants for histological examination 23
   3.3. Specific methods (I-IV) 24

4. RESULTS 25
4.1. The development of mouse mandibular first and second molars *in vitro* 25
4.1.1. The developmental stages at the start of culture (E18) (I, II, III, IV) 25
4.1.2. Stages of development after culture 25
4.1.2.1. Stages of development after 5 days of culture (E18+5; stereomicroscopic findings) (IV) *25*
4.1.2.2. Stages of development after 10/12 days of culture (E18+10, E18+12; stereomicroscopic and histological findings) (I, II, IV) *25*
4.1.3. Apoptosis in developing first and second molars (II, IV) 26
4.1.4. Cell proliferation in developing first and second molars (II, IV) 27
4.1.5. Amelogenin expression in developing first molar (IV) 27
4.2. Effects of environmental toxicants and fluoride on cultured mouse mandibular first and second molar development 27
4.2.1. Effects of 7,12-dimethylbenz[a]anthracene (DMBA) (I) 27
4.2.1.1. Morphological findings in the first molar *27*
4.2.1.2. Morphological findings in the second molar *28*
4.2.2. Effects of tributyltin (TBT) (II, III) 28
4.2.2.1. Morphological findings in the first molar (II) *29*
4.2.2.2. Effect on apoptosis and cell proliferation in the first molar (II) *31*
4.2.2.3. Morphological findings in the second molar (II) *31*
4.2.2.4. Effect on apoptosis and cell proliferation in the second molar (II) *31*
4.2.2.5. Effects on gene expression in the first molar (III) *32*
4.2.3. Effects of sodium fluoride (NaF) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (IV) 33
4.2.3.1. Stereomicroscopic and histological findings *33*
4.2.3.2. Amelogenin expression in the first molar *35*
4.2.3.3. Apoptosis in the first and second molars *35*
4.2.3.4. Cell proliferation in the first and second molars *35*

5. DISCUSSION 37
5.1. General aspects (I, II, III, IV) 37
5.2. Methodological considerations (I, II, III, IV) 37
5.3. Clinical relevance of exposure *in vitro* 38
5.3.1. Exposure route and timing (I, II, III, IV) 38
5.3.2. The effect on size and shape of tooth (I, II, IV) 39
5.4. The effects on dentin and enamel formation and mineralization (I, II, IV) 40
5.5. Mechanistic aspects 41
5.5.1. The role of epithelial-mesenchymal interactions (I, II, IV) 41
5.5.2. The effects at the cellular level: apoptosis and cell proliferation (II, IV) 41
5.5.3. The effect at the protein level: amelogenin expression (IV) 42
5.5.4. The effects on gene expression (III) 43

6. CONCLUSIONS 45

REFERENCES 46

ACKNOWLEDGEMENTS 61

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles, which are referred to in the text by their Roman numerals.

I Peltonen, E., Lukinmaa, P.-L., Sahlberg, C., Partanen, A.-M., Kiukkonen, A., Alaluusua, S. 7,12-Dimethylbenz[a]anthracene Interferes with the Development of Cultured Mouse Mandibular Molars. *Toxicological Sciences* 2006; 92(1), 279-285.
Doi: 10.1093/toxsci/kfj199
Oxford University Press and the Society of Toxicology are acknowledged for the permission to reprint the article.

II Salmela, E., Sahlberg, C., Alaluusua, S., Lukinmaa, P.-L. Tributyltin impairs dentin mineralization and enamel formation in cultured mouse embryonic molar teeth. *Toxicological Sciences* 2008; 106(1), 214-222.
Doi: 10.1093/toxsci/kfn156
Oxford University Press and the Society of Toxicology are acknowledged for the permission to reprint the article.

III Salmela, E., Alaluusua, S., Sahlberg, C., Lukinmaa, P.-L. Tributyltin alters *osteocalcin*, *matrix metalloproteinase 20*, and *dentin sialophosphoprotein* gene expression in mineralizing mouse embryonic tooth *in vitro*. *Cells Tissues Organs.* (in press).
S. Karger AG is acknowledged for the permission to reprint the article.

IV Salmela, E., Lukinmaa, P.-L., Partanen, A.-M., Sahlberg, C., Alaluusua, S. Combined effect of fluoride and 2,3,7,8-tetrachlorodibenzo-p-dioxin on mouse dental hard tissue formation *in vitro*. *Arch. Toxicol.* 2010 (Epub on Nov 28th, in press)
Doi: 10.1007/s00204-010-0619-4
Springer-Verlag is acknowledged for the permission to reprint the article.

ABBREVIATIONS

Ah	Aryl hydrocarbon
AhR	Aryl hydrocarbon receptor
AI	Amelogenesis imperfecta
Alpl	The gene encoding tissue non-specific alkaline phosphatase
AMELX	Amelogenin
AMTN	Amelotin
APECED	Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy
ARNT	Aryl hydrocarbon receptor nuclear translocator
BMP	Bone morphogenetic protein
BP	Benzo[a]pyrene
BrdU	5'-bromo-2'-deoxyuridine
BSP	Bone sialoprotein
Ca	Calcium
CO_2	Carbon dioxide
CYP	Cytochrome P450
DD	Dentin dysplasia
DGI	Dentinogenesis imperfecta
DGP	Dentin glycoprotein
Dlx	Distal-less homeobox
DMBA	7,12-dimethylbenz[a]anthracene
DMP1	Dentin matrix protein-1
DNA	Deoxyribonucleic acid
DPP	Dentin phosphoprotein, or phosphophoryn
DSP	Dentin sialoprotein
DSPP	Dentin sialophosphoprotein
E	Embryonic day
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
ENAM	Enamelin
F	Fluoride
FAM83H	Family with sequence similarity, member H
FGF	Fibroblast growth factor
GAG	Glycosaminoglycan
GGT	Gamma glutamyl transferase
Gla	Gamma-carboxylated
H/W	Han/Wistar (rats)
HA	Hydroxyapatite
HE	Hematoxylin and eosin
HERS	Hertwig's epithelial root sheath
IARC	The International Agency for Research on Cancer
IEE	Inner enamel epithelium
KLK4	Kallikrein4
LE	Long-Evans (rats)
Lhx	Lim homeobox protein
MEPE	Matrix extracellular phosphoglycoprotein
MIH	Molar-incisor hypomineralization

MMP	Matrix metalloproteinase
MMP-2	Matrix metalloproteinase 2
MMP-20	Matrix metalloproteinase 20
mRNA	Messenger ribonucleic acid
Msx	Hox8; Msh (muscle segment homeobox) drosophila homolog
NaF	Sodium fluoride
NCP	Noncollagenous matrix proteins
NMRI	The Naval Medical Research Institute (mouse strain)
OCN	Osteocalcin
OEE	Outer enamel epithelium
OI	Osteogenesis imperfecta
OPN	Osteopontin
OTC	Organic tin compound
PAH	Non-halogenated polycyclic aromatic hydrocarbon
Pax9	Paired box gene 9
PCB	Polychlorinated biphenyls
PCDD	Polychlorinated dibenzo-*p*-dioxin
PCDF	Polychlorinated dibenzofuran
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PG	Proteoglycans
PHEX	Phosphate-regulating gene with homologies to endopeptidases on X chromosome
PN	Postnatally
Ppm	Parts per million
QPCR	Quantitative PCR
RNase	Ribonuclease
RNA	Ribonucleic acid
Runx2	Runt related transcription factor 2
SA	Sialic acid
Shh	Sonic hedgehog
SIBLING	Small integrin-binding ligand, N-linked glycoprotein
TBT	Tributyltin
TBTCl	Tributyltin chloride
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TDI	Tolerable daily intake
TGFβ	Transforming growth factor beta
TNAP	Tissue non-specific alkaline phosphatase
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling
USEPA	U.S. Environmental Protection Agency
Wnt	Wingless
XLH	Human X-linked hypophosphatemia

ABSTRACT

Dioxins and dioxin-like compounds, organic toxicants ubiquitous in our environment, are known to impair tooth development, especially dental hard tissue formation. For example, exposure of children to the most toxic congener of these compounds, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), has been found to correlate with the occurrence of developmental enamel defects and missing teeth since the dioxin accident in Seveso, Italy, in 1976. Enamel defects have also been detected more often in Slovakian children exposed to high concentrations of polychlorinated biphenyls (PCB) than in children exposed to lower concentrations. Although, concentrations prevailing in the environment may be sufficiently high to impair tooth development: exposure to dioxins and dioxin-like compounds in infancy through breast-feeding has been connected to molar-incisor hypomineralization of the dental enamel (MIH). Results of previous *in vivo* and *in vitro* studies with TCDD support the clinical observations. Clinical studies also suggest that maternal smoking during pregnancy can reduce the child's tooth size. One of the main components of tobacco smoke is the group of non-halogenated polycyclic aromatic hydrocarbons (PAHs), a representative of which is 7,12-dimethylbenz[a]anthracene (DMBA). Tributyltin (TBT), an organic tin compound, has been shown to impair bone formation and mineralization in experimental animals but its effect on tooth development had not been studied earlier. In addition to exposure to organic toxicants, a well-established cause for enamel hypomineralization is excess fluoride intake. Humans are exposed to many environmental toxicants although usually in very low and mostly insignificant amounts. However, additive and even potentiative effects of various chemicals are possible.

The principal aim of this thesis project was to examine *in vitro* if, in addition to dioxins and dioxin-like compounds, other organic environmental toxicants, like PAHs and organic tin compounds, have adverse effects on tooth development, specifically on formation and mineralization of dentin and enamel. The second main aim was to investigate *in vitro* if fluoride could intensify the manifestation of the detrimental developmental dental effects elicited by dioxins and dioxin-like compounds. In order to address these goals, mandibular first and second molar tooth germs of E18 NMRI mouse embryos were cultured in a Trowell-type organ culture and exposed to DMBA, TBT, and sodium fluoride (NaF) and/or TCDD at various concentrations during the secretory and mineralization stages of development. Morphological changes were studied from photographs of whole tooth explants and histological tissue sections. Tissue sections of explants were stained by TUNEL, for apoptosis, and by BrdU-labeling, for cell proliferation, to determine the effects of TBT, NaF and TCDD. The presence of amelogenin was analyzed by immunohistochemistry from sections of NaF and TCDD exposed first molars. The expression of genes related to mineralization of dental hard tissues in control and TBT exposed first molars were compared by real-time quantitative PCR (QPCR) and *in situ* hybridization.

Exposure to DMBA and TBT significantly reduced the size of tooth germs and impaired deposition and mineralization of dentin and enamel. TBT exposure moderately enhanced apoptotic cell death mainly in epithelial tissues in the first molar and increased the frequency of BrdU-labeled nuclei in the second molar. *In situ* hybridization results showed that exposure of tooth germs to TBT decreased *osteocalcin* (*Ocn*) gene expression in odontoblasts but increased its expression in the epithelial compartment of the tooth. Using QPCR, the net effect in the whole tooth was an increase in *Ocn* expression. TBT exposure also reduced *matrix metalloproteinase 20 (Mmp-20)* expression in both ameloblasts and odontoblasts. The

effect of TBT on *dentin sialophosphoprotein* (*Dspp*) expression varied but both QPCR experiments and *in situ* hybridization showed a decreasing trend. Simultaneous exposure to NaF and TCDD at concentrations that alone had no, or a barely detectable, effect on tooth development, significantly impaired dentin mineralization and enamel matrix deposition. The enamel consistently stained darker after NaF and TCDD exposure compared to control histological tissue sections stained with hematoxylin and eosin (HE), suggesting retention of enamel matrix proteins and thereby hypomineralization. Immunohistochemical analysis revealed that the combined exposure modified amelogenin expression by odontoblasts. Apoptosis of secretory ameloblasts or odontoblasts was not enhanced, but simultaneous exposure to NaF and TCDD significantly reduced cell proliferation in the second molar. The second molars exposed to both NaF and TCDD in combination also seemed to be smaller than controls.

For ameloblasts to become secretory, odontoblasts must have laid down a thin layer of predentin that should have commenced mineralization. Therefore, these results suggest that the effect of DMBA and TBT, and the combined effect of NaF and TCDD, is directed primarily to odontoblasts or dentin mineralization. Decreased *Ocn*, *Mmp-20* and *Dspp* expressions in odontoblasts after TBT-exposure may indicate delayed cell differentiation or alternatively, TBT may specifically decrease the expression of genes involved in the mineralization of dentin. While TBT-induced decreased *Mmp-20* expression in ameloblasts may impair enamel mineralization, the coincident reduction of *Mmp-20* and *Dspp* expressions in odontoblasts may potentiate a delay in dentin mineralization. The mechanism behind the detrimental effects of exposure to higher concentrations of the toxicants studied may involve increased apoptosis. However, very low concentrations of NaF and TCDD, or their combination, did not enhance apoptosis of secretory ameloblasts or odontoblasts. Thus, the present results do not suggest a role for apoptosis in impairing dental hard tissue formation after exposure to organic environmental toxicants and fluoride, especially at low concentrations.

This thesis work showed that, in addition to dioxins and dioxin-like compounds, other organic environmental toxicants also have detrimental effects on dental hard tissue formation and that fluoride can potentiate the harmful effect of TCDD. Dioxins, dioxin-like compounds, PAHs and organic tin compounds are all liposoluble and can be transferred to the infant by breast-feeding. At the same time, children get fluoride by early use of fluoride toothpaste. Because developing teeth are susceptible to each of these compounds, they could interfere with the formation of dental hard tissues not only separately but also in combination. Thus, the findings of the present study may have clinical significance. However, since this thesis work is based on *in vitro* experiments of mouse tissues, its results cannot as such be directly applied to humans and further research would be required to make these findings clinically significant.

1. REVIEW OF THE LITERATURE

1.1. Tooth development

Tooth development closely resembles the development of other epithelial appendages such as hair follicles, mammary glands and kidneys. However, a characteristic feature of tooth development is the formation of hard tissues, ectomesenchymal-derived dentin and epithelium-derived enamel. Dental hard tissues also include cementum.

1.1.1. Initiation and morphogenesis

Teeth develop as a result of sequential and reciprocal epithelial-mesenchymal interactions (Thesleff, 2003). Inductive interactions regulate tooth development during all stages from the initiation to the early phases of matrix secretion. The main signaling proteins involved in the mediation of these interactions are members of the fibroblast growth factor (FGF), bone morphogenetic protein (BMP) belonging to the TGFβ-superfamily, sonic hedgehog (Shh), and wingless (Wnt) families of growth factors (Jernvall and Thesleff, 2000).

Tooth development begins with the induction of odontogenic mesenchyme by BMPs and FGFs from the dental epithelium (Thesleff, 2006). Odontogenic mesenchymal cells derive from the neural crest in the midbrain region (Imai et al., 1996). In mouse, tooth formation begins at E10. Reciprocal signal molecules, including activin, FGF, BMP, Wnt and Shh shifting between the mesenchyme and the epithelium, regulate the thickening of the oral ectoderm and formation of the dental placode at the site of each future tooth family (in mouse at E11). As development proceeds, the placodal signals regulate budding of the epithelium, around which neural crest-derived ectomesenchymal cells condense (E13). Morphogenesis continues through the cap stage (E14-15), during which the epithelium grows and folds to surround the dental papilla, to the bell stage (E16-18), during which the basic cuspal morphology is completed. A schematic presentation of mouse mandibular first molar tooth development is shown in Figure 1.

Growth and folding of the dental epithelium is controlled by several transient signaling centers in the epithelium. The first signaling centers appear in the dental placodes when epithelial budding begins. The enamel knot signaling centers appear at the bud-cap (primary) and bell stages (secondary). Mesenchymal signal molecules, in particular BMP4, are key regulators of formation of enamel knots (Bei et al., 2000; Jernvall et al., 1998). The enamel knot cells express at least ten different signaling molecules belonging to the four growth factor families. Signals affect both epithelial and mesenchymal cells (Jernvall et al., 1994; Jernvall and Thesleff, 2000). The signal molecules change gene expression in their target cells by many important transcription factors. These include e.g. Msx, Dlx, and Lhx families of transcription factors at the initiation stage, and Msx1, Pax9, and Runx2 at the bud-cap stages (Thesleff, 2006).

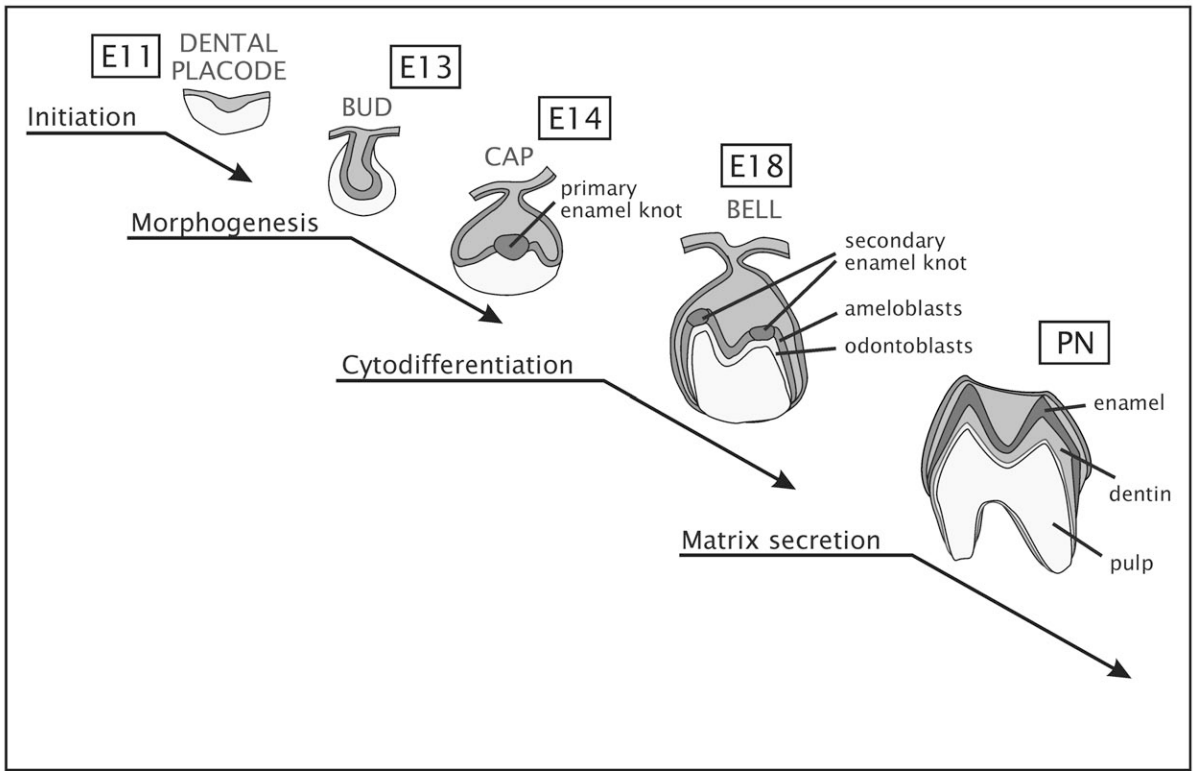

Figure 1. Schematic presentation of mouse mandibular first molar tooth development (frontal view). The first sign of tooth development is the thickening of oral epithelium (gray) at the site of a future tooth (E11). The epithelium forms a bud, around which neural crest-derived ectomesenchymal cells (white) condense (E13). Morphogenesis continues through the cap stage (E14) to the (late) bell stage (E18) when the basic cuspal morphology has been completed. Enamel knots control growth and folding of the epithelium. Differentiation of the ectomesenchymal odontoblasts and epithelial ameloblasts has started. Postnatally (PN), odontoblasts have laid down a thick (pre)dentin layer. Dentin mineralization and the subsequent formation of enamel by ameloblasts have proceeded throughout the crown and root formation is ongoing.

1.1.2. Differentiation of the dental cells

At the late bell stage (E18.5) morphogenesis has been completed and terminal differentiation of the odontoblasts and ameloblasts starts to proceed from the tips of the cusps in the cervical direction. Ameloblasts form from the inner enamel epithelium (IEE) of the enamel organ and the underlying mesenchymal cells of the dental papilla differentiate into odontoblasts. Signals from the preameloblasts to the preodontoblasts, across the epithelial basement membrane between these cells, initiate the final series of reciprocal signaling (Ruch et al., 1995; Thesleff and Hurmerinta, 1981; Figure 2). It is supposed that BMP-2 from the IEE stimulates the differentiation of odontoblasts (Begue-Kirn et al., 1992; Åberg et al., 1997). Interaction between preodontoblasts and the basement membrane also plays an important role (Thesleff and Hurmerinta, 1981).

During terminal differentiation, the preodontoblasts polarize, that is, their nuclei are positioned away from the IEE, become odontoblasts and begin to produce predentin matrix (Figure 2). Odontoblasts have been shown to express low amounts of amelogenin, the main enamel matrix protein, prior to abundant amelogenin expression by secretory ameloblasts (He et al., 2010; Nagano et al., 2003; Papagerakis et al., 2003). He et al. (2010) suggest that amelogenin expressed by odontoblasts may play a role in inducing presecretory ameloblasts

further to differentiate to secretory stage cells. Tompkins et al. (2005), for one, hypothesize that an amelogenin isoform [A-4] produced by secretory odontoblasts may normally delay conversion of preameloblasts to secretory ameloblasts until a sufficiently thick layer of dentin is produced, whereas another isoform [A+4] stimulates odontoblast development and dentin formation thereby enhancing differentiation of ameloblasts.

However, the initial mineralization of predentin is a requirement for the preameloblasts to become secreting ameloblasts (He et al., 2010; Tompkins, 2006; Figure 2). The molecules regulating differentiation of ameloblasts also include BMPs produced by the mesenchymal cells (Wang et al., 2004). In a recent study, deletion of an RNase, Dicer-1, which processes micro-RNAs in epithelium caused impaired ameloblast differentiation and enamel formation (Michon et al., 2010). Micro-RNAs are short, non-coding RNAs that participate in post-transcriptional regulation of mRNA, and are suggested to modulate the main morphogenetic signaling pathways regulating, for example, dental cell differentiation (Michon et al., 2010; Singh et al., 2008). Also basement membrane may have an important function in differentiation of ameloblasts (Chen et al., 2009; He et al., 2010). Thus, cell-matrix interactions, signaling molecules and micro-RNAs are likely to be needed for ameloblast differentiation.

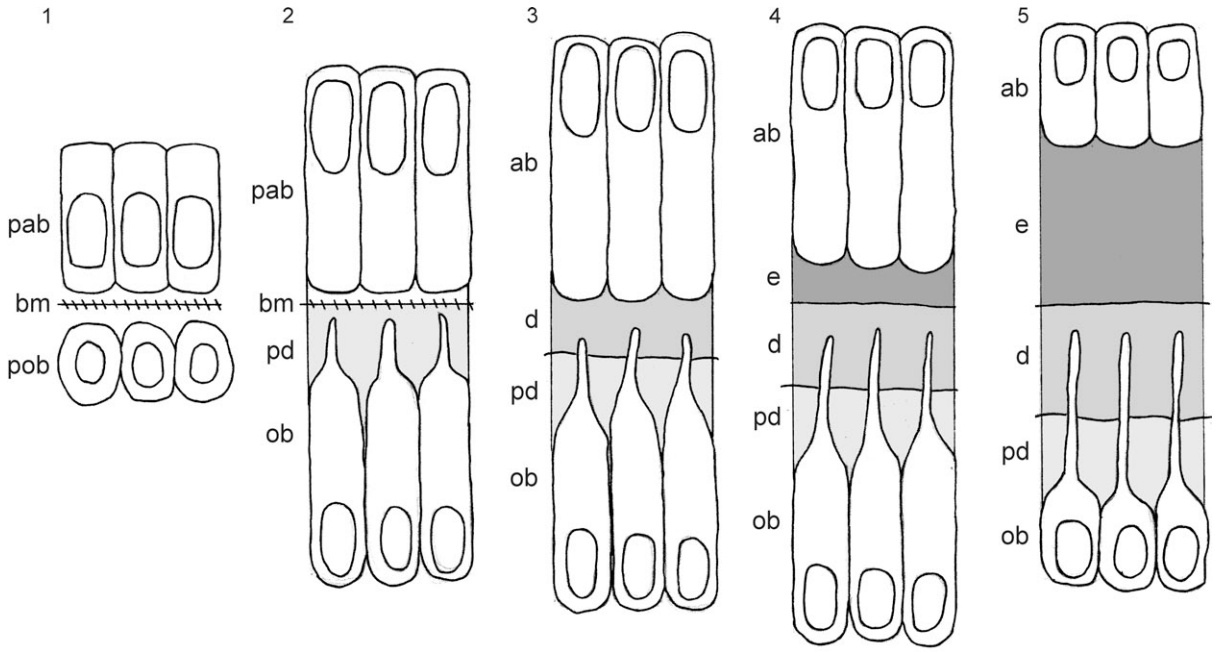

Figure 2. Cartoon schematically representing differentiation of odontoblasts and ameloblasts, and formation of dentin and enamel. (1) Signals from preameloblasts across the basement membrane induce preodontoblasts to differentiate into odontoblasts. Interaction between preodontoblasts and the basement membrane also plays an important role. (2) Polarized odontoblasts have begun to produce predentin matrix, receded and left behind cell processes within dentinal tubules. Preameloblasts are lengthening. (3) Basement membrane has disintegrated, and first-formed predentin has mineralized into dentin, which is a requirement for preameloblasts to become secreting ameloblasts. (4) Ameloblasts have secreted enamel matrix onto the surface of the mineralized dentin and shortened. The latest formed layer of predentin remains permanently unmineralized. (5) When the full thicknesses of dentin and enamel matrices have been deposited, ameloblasts have lost their elongated shape and shifted into the maturation stage. Odontoblasts have also shortened. pab, preameloblasts; bm, basement membrane; pob, preodontoblasts; pd, predentin; ob, odontoblasts; ab, ameloblasts; d, dentin; e, enamel.

Peripheral to the IEE, the stratum intermedium, stellate reticulum, and the outer enamel epithelium are located. The most superficial and mesenchymal part of the tooth germ, the dental follicle, surrounding the tooth epithelium and the mesenchymal papilla, gives rise to the periodontal structures: the cementum, the periodontal ligament, and alveolar bone.

According to the classic theory, cementoblasts differentiate from the dental follicle cells during root formation (Diekwisch, 2001). The IEE forms a cervical loop with the outer enamel epithelium (OEE), and this two cell layer loop is called Hertwig's epithelial root sheath (HERS). HERS cells are suggested to trigger odontoblast differentiation and dentin formation during root formation, and to regulate the differentiation of cementoblasts (Thomas and Kollar, 1989; Zeichner-David, 2006). The HERS cells deposit basement membrane molecules and enamel proteins on the root dentin, which serves to direct the migration of precementoblast cells, and which may induce differentiation of the dental follicle cells into cementoblasts (Zeichner-David, 2006). However, recently it has been suggested that acellular cementum on the cervical half of the root is formed by mesenchymally transformed epithelial cells of HERS and cellular cementum on the apical half of the root by cells that derive from the dental follicle (Lézot et al., 2000; Zeichner-David et al., 2003).

1.1.3. Formation and mineralization of the dental hard tissues

Dental hard tissues include the enamel, dentin and cementum, produced by ameloblasts, odontoblasts and cementoblasts, respectively.

1.1.3.1. Predentin and mineralized dentin

Composition, formation, and mineralization. - Mature dentin is composed of approximately 70% inorganic material, 20% organic material, and 10% water by weight. The inorganic component of dentin consists of (substituted) hydroxyapatite (HA; $Ca_{10}[PO_4]_6[OH]_2$) and the organic phase is composed of about 90% collagen. Dentinogenesis is a continuous process of matrix deposition throughout the life of a tooth. Odontoblasts firstly secrete an unmineralized layer of dentin matrix called predentin that is composed mainly of type I collagen fibrils. Predentin gradually mineralizes into dentin as various noncollagenous matrix proteins (NCP) and HA are incorporated at the mineralization front. The latest formed layer of predentin remains permanently unmineralized. Predentin is thickest during tooth development and diminishes in thickness thereafter. Primary dentinogenesis occurs during development, whereas secondary dentin is secreted throughout the life of the tooth. Tertiary (reactionary or reparative) dentin is synthesized in response to injury. During predentin matrix deposition, odontoblasts recede and leave behind cell processes within dentinal tubules (Figure 2). After the completion of tooth development, the dentin layer next to enamel represents the first-formed predentin (mantle dentin).

The dynamic process of biomineralization involves interplay between a number of molecules, including type I collagen, NCPs, and proteoglycans. During the transformation of predentin to dentin, plate-like apatite crystals are initiated and grow within and around collagen fibrils. Calcium and phosphate ions are transferred from the vascular bed across odontoblasts by specific ion-transporting mechanisms into the organic matrix of dentin (Linde and Lundgren, 1995). Multiple globular mineral foci (calcospherites) grow and coalesce with the adjacent foci to form a relatively uniform mineralization front. Among NCPs, the sialic acid (SA)-rich proteins are of particular interest, because they appear to be regulators of the mineralization process in dentin. The SIBLING (Small Integrin-Binding LIgand, N-linked Glycoprotein)

protein family includes four SA-rich proteins; dentin sialophosphoprotein (DSPP), dentin matrix protein-1 (DMP1), osteopontin (OPN) and bone sialoprotein (BSP), and matrix extracellular phosphoglycoprotein (MEPE), which is not acidic in character (Fisher et al., 2001). Their genes have been mapped to human chromosome 4q (Fisher et al., 2001). Besides SIBLING proteins, proteoglycans, osteocalcin and tissue non-specific alkaline phosphatase also play roles in dentin mineralization.

Dentinogenesis and osteogenesis resemble each other in many ways. Dentin forming odontoblasts and bone forming osteoblasts are both mesenchymal cells. However, odontoblasts originate from neural crest derived ectomesenchyme whereas osteoblasts may originate from both ectomesenchyme and mesoderm. The formation of both dentin and bone starts as the deposition of collagen-rich extracellular matrix, predentin and osteoid respectively, which undergo mineralization to dentin and bone. Bone and dentin share the expression of numerous NCPs. Since some of these proteins and their functions have been studied mostly in bone, such studies are referred to here.

Collagen. **-** Collagens are the main constituents of the extracellular matrix (ECM). Collagens are grouped into fibril-forming and non-fibril-forming collagens. At least 29 types of collagens are currently identified. Type I collagen is a fibril-forming collagen, which means that the molecules self-assemble into larger collagen aggregates, the fibrils. The type I collagen molecule is a heterotrimer of two identical α1 and one α2 polypeptide chains forming a triple-helical structure. A minor part of type I collagen is synthesized as a homotrimer of three α1 chains. Collagen undergoes extensive post-translational modification.

SIBLING proteins. **-** Dentin sialophosphoprotein (DSPP) is considered highly tooth-specific but low levels of *Dspp* expression, about one in four-hundred that of dentin, have been found in bone as well (Qin et al., 2002). It is mainly expressed in odontoblasts throughout all phases of primary and secondary dentinogenesis but transient expression has also been observed in preameloblasts (D'Souza et al., 1997). DSPP is post-translationally cleaved to NH_2-terminal dentin sialoprotein (DSP) and COOH-terminal dentin phosphoprotein, or phosphophoryn (DPP) domains, which are the predominant NCPs of dentin. The existence of a third DSPP domain, dentin glycoprotein (DGP), has been demonstrated in pig (Yamakoshi et al., 2005). Highly phosphorylated DPP contains large amounts of aspartic acid and phosphoserine. DSP is a sialic acid-rich glycosylated protein. DPP has a strong affinity for Ca^{2+} and it significantly promotes the growth of HA crystals when bound to collagen fibrils *in vitro* (Saito et al., 1997). Even though earlier studies have shown that DSP has little or no effects on *in vitro* mineralization (Boskey et al., 2000), Suzuki et al. (2009) suggested that DSP predominantly regulates initiation of dentin mineralization, whereas DPP is primarily involved in the maturation of mineralized dentin. Teeth of *Dspp* knockout mice display a widened predentin zone and defective dentin mineralization (Sreenath et al., 2003). Since all known nonsyndromic genetic diseases of human dentin exclusively involve the *DSPP* gene (Barron et al., 2008; McKnight et al., 2008), it is implicated as a critical molecule in converting predentin to dentin.

Dentin matrix protein 1 (DMP1) has a large number of phosphoseryl residues and it is a highly acidic protein that is present predominantly in dentin, cementum and bone. DMP1 expression is most dominant in the early phases of primary dentin formation, declining considerably as dentinogenesis proceeds (D'Souza et al., 1997; Lu et al., 2007); it is also expressed in ameloblasts at the maturation phase (D'Souza et al., 1997). DMP1 is proteolytically processed to smaller fragments [NH_2-terminal (37 kDa) and COOH-terminal (57 kDa)] in dentin and bone (Qin et al., 2003). Furthermore, a glycanated form (DMP1-PG)

coexists with the non-glycanated 37-kDa fragment in the ECM of bone (Qin et al., 2006). The proteolytic process may involve the same proteinase that cleaves DSPP (Qin et al., 2003). Like DPP, the highly phosphorylated COOH-terminal (57 kDa) fragment of DMP1 is distributed primarily in the inorganic phase (Huang et al., 2008), and since it has a high capacity to bind calcium it therefore has been suggested to initiate HA formation (Gajjeraman et al., 2007; He et al., 2003). Conversely, the NH_2-terminal domain inhibits HA crystallization (Gajjeraman et al., 2007). *Dmp1* null mice show profound defects in the mineralization of dentin (Ye et al., 2004). Further, malformation of the dentinal tubular network has been observed (Lu et al., 2007). The tooth phenotype resembles that in *Dspp* null mice and shares some features of the human dentinogenesis imperfecta type III (Sreenath et al., 2003). Mutations in the *DMP1* gene result in autosomal-recessive hypophosphatemic rickets in humans (Feng et al., 2006). *In vitro* overexpression of *Dmp1* induces differentiation of mesenchymal cells to odontoblast-like cells and enhances mineralization (Narayanan et al., 2001). Since DSPP is reduced in *Dmp-1* null mutant mice at both mRNA and protein levels, DMP1 has been implicated in regulation of *Dspp* expression during dentinogenesis (Ye et al., 2004). In line, in another study, *in vitro* overexpression of *Dmp1* induced *Dspp* expression (Narayanan et al., 2001). It may also act as a transcriptional component for activation of osteocalcin (Narayanan et al., 2003). Thus, Narayanan et al. (2003) proposed that DMP1 has a dual function, as a transcription factor located in the nucleus during early differentiation of osteoblasts and as an extracellular matrix protein that initiates mineralization. The export of DMP1 from the nucleus during the maturation of osteoblasts has been found to occur in response to a stimulus from influx of calcium ions into the nucleus (Narayanan et al., 2003).

PHEX protein (encoded by phosphate-regulating gene with homologies to endopeptidases on X chromosome) has been suggested to be the enzyme responsible for the proteolytic processing of DSPP and DMP1 (Qin et al., 2003). However, in a recent study PHEX protein was shown not to be responsible for the proteolytic cleavage of these proteins (Zhang et al., 2010). Three isoforms of bone morphogenetic protein 1 (BMP1) have been observed to cleave intact DSPP into DSP and DPP fragments and process also DMP1 (von Marschall and Fisher, 2010). Further, both matrix metalloproteinase 20 (MMP-20) and matrix metalloproteinase 2 (MMP-2) perform cleavages that separate DSP and DGP (Yamakoshi et al., 2006). MMP-20 also generates a series of DSP-positive cleavage products and MMP-2 makes cuts within the DGP domain (Yamakoshi et al., 2006). Accordingly, inhibition of MMP activity impairs dentin mineralization in cultured mouse embryonic molars (Bourd-Boittin et al., 2005). DPP also undergoes degradation as dentin matures (Masters, 1985) and intact DPP is virtually absent from mature human teeth (Chang et al., 1996). It is questionable regarding whether the NCPs in dentin are degraded to create space for increased mineralization.

OPN is a phosphorylated glycoprotein that strongly inhibits *in vitro* growth of apatite crystals (Hunter et al., 1996). Accordingly, *Opn* knockout mice have increased mineral content and maturity in bone (Boskey et al., 2002). The amount of OPN in bone is about 70 times that of dentin (Butler et al., 2003). Also OPN has been shown to be processed to two fragments after translation (Chen et al., 2008).

BSP is a phosphorylated and sulfated glycoprotein that *in vitro* acts as a nucleator of the initial apatite crystals (Hunter and Goldberg, 1993). Binding of BSP to type I collagen increases its HA-nucleating potency (Baht et al., 2008). The quantity of BSP in dentin is equal to or less than that of bone (Fujisawa et al., 1993; Qin et al., 2001). Intact protein is processed within the mouse odontoblastic cells before secretion (Chen et al., 2008).

Matrix extracellular phosphoglycoprotein (MEPE) is believed to be multifunctional, having roles in processes such as cell adhesion, phosphate homeostasis, and mineralization (Argiro et al., 2001; David et al., 2011; Wang et al., 2011). MEPE is expressed by both odontoblasts and osteoblasts (osteocytes) (Chen et al., 2008; Nampei et al., 2004), and it has been suggested to inhibit bone formation and mineralization (Gowen et al., 2003). Chen et al. (2008) observed that MEPE is also post-translationally processed to three major fragments in mouse odontoblastic cells. These fragments have been shown to have different functions, as the COOH-terminal fragment inhibits the mineralization process *in vitro* whereas a midterminal fragment stimulates bone formation *in vitro* and *in vivo* (Hayashibara et al., 2004; Rowe et al., 2005).

Other proteins involved in dentin mineralization. **-** Proteoglycans (PG) are also proposed to play a key role in the mineralization process of dentin. PGs belong to a family of glycoconjugates that contain one or more glycosaminoglycans (GAG) covalently attached to a protein core. In predentin, small leucine-rich PGs such as biglycan and decorin with only one or two chondroitin sulfate or dermatan sulfate GAG side chains are the predominant PGs. They are suggested to organize type I collagen into a more fibrillar form near the mineralization front in order to induce the proper crystal formation along the collagen fibrils and inside the fibrils (Embery et al., 2001). Both decorin and biglycan have the capacity to bind collagen molecules (Pogany et al., 1994; Schonherr et al., 1995a; 1995b). The GAG side chains are known to bind calcium and interact with HA (Embery et al., 1998). During the transition from predentin to dentin, PGs increase the binding affinity for HA probably due to different binding affinities of various GAG moieties (Milan et al., 2004). Dentinal PGs are predominantly chondroitin sulphate rich decorin and biglycan (Milan et al., 2004). The teeth of newborn biglycan- and decorin-null mice show defective dentin mineralization (Goldberg et al., 2005). Further, the coalescence of calcospherites in the dentin of DSPP/biglycan double knockout mice was severely reduced compared to that of *Dspp*$^{-/-}$ mice (Haruyama et al., 2009). On the other hand, the width of the predentin layer in incisors of DSPP/decorin double knockout mice dramatically decreased as compared to *Dspp*$^{-/-}$ mice (Haruyama et al., 2009). Since *Dspp*$^{-/-}$ mice demonstrate an increased deposition of biglycan and decorin in the predentin zone (Sreenath et al., 2003), the conversion of predentin to dentin at the mineralization front in *Dspp*$^{-/-}$ mice may be regulated positively by biglycan and negatively by decorin (Haruyama et al., 2009). Accordingly, biglycan increases HA formation *in vitro* (Boskey et al., 1997) and decorin binds to the gap region in the collagen fibril, which is believed to block initiation of mineralization (Hoshi et al., 1999).

Osteocalcin (Ocn) is also a major non-collagenous protein in bone, dentin and cementum (Hauschka et al., 1989). It is a gamma-carboxylated protein (gla-protein) that binds to apatite crystals with high affinity (Poser and Price, 1979). *Ocn* is strongly expressed by mature odontoblasts depositing predentin before it starts to mineralize to dentin (Bidder et al., 1998; Bleicher et al., 1999; Bronckers et al., 1987). It is not or weakly detected in predentin and strongly evident in mineralizing dentin (Bronckers et al., 1998; Gorter De Vries et al., 1987). Thus far, the exact role of Ocn in bone and tooth has not been defined; however, Ducy et al. (1996) suggested that it restricts bone formation without affecting bone resorption or mineralization. Boskey et al. (1998) showed that bone maturation is impaired in the *Ocn* knockout animals, which implies a role for Ocn in mineral maturation. Price et al. (1982) proposed that Ocn may be responsible for preventing excessive mineralization of the bone growth plate, but Murshed et al. (2004) did not find any mineralization defects in bones of mice overexpressing *Ocn* in osteoblasts. Serum Ocn concentration has been observed to be increased in many patients with metabolic bone diseases (Price et al., 1980). On the other

hand, no histological changes in tooth formation have been detected in *Ocn* knockout mice (Bronckers et al., 1998). However, hyper-expression of *Ocn* has been observed by QPCR in first molars of Hyp mice (Onishi et al., 2005), and this hyper-expression was suggested to result in hypo-mineralization of dentin (Ogawa et al., 2006). The Hyp mouse is a murine homologue of human X-linked hypophosphatemia (XLH) (vitamin D resistant rickets). The genetic defect underlying XLH is mutations in the *Phex* gene (Econs and Francis, 1997). Histopathological studies of XLH patients' teeth have revealed hypomineralization of dentin indicated by abundant unmineralized interglobular dentin, broad predentin and irregular dentinal tubules (Abe et al., 1988; Seow et al., 1989). Onishi et al. (2005) proposed that *Ocn* is hyper-expressed in odontoblasts of Hyp mice, since *Ocn* mRNA was detected by *in situ* hybridization in odontoblasts alone. However, they did not find differences in the distribution pattern or the amount of *Ocn* mRNA between Hyp and wild-type mice by *in situ* hybridization. Furthermore, exposure of cultured hamster tooth explants to exogenous Ocn impaired the formation of mineralized dentin and enamel (Bronckers et al., 1998). Ocn is also known to inhibit hydroxyapatite crystal nucleation and growth *in vitro* (Hunter et al., 1996; Romberg et al., 1986). Vitamin D (as 1,25-dihydroxyvitamin D3) induces the synthesis of osteocalcin by promoting the transcription of its gene (Bronckers et al., 1998; Lian et al., 1989).

Among the four existing isoforms of alkaline phosphatase, the tissue non-specific alkaline phosphatase (TNAP, coded by *Alpl* gene) is expressed for example by osteoblasts, odontoblasts and ameloblasts (Hoshi et al., 1997; Hotton et al., 1999). During dentin mineralization, the junction between predentin and dentin is particularly rich in TNAP protein (Hotton et al., 1999). TNAP has been proposed to dephosphorylate phosphoproteins and thereby make inorganic phosphate available for HA crystals (Whyte, 1994). It controls bone mineralization (Harmey et al., 2004) and is also supposed to stimulate dentin mineralization (Beertsen et al., 1999). TNAP-deficient mice show delayed mineralization of the mantle dentin (first-formed predentin) (Beertsen et al., 1999).

1.1.3.2. Enamel

Composition, formation, and mineralization. - Enamel consists of approximately 96% mineral and only 0.5-2% organic material (Deakins and Volker, 1941). The inorganic component of enamel is HA substituted with components such as carbonate and fluoride ions. HA crystallites form enamel rods, formerly called enamel prisms. As the final step of epithelial-mesenchymal interactions instructing early tooth development, enamel formation (amelogenesis) by ameloblasts begins only after mineralization of predentin has started (Tompkins, 2006). An optimal serum calcium level is important for initial dentin mineralization and proper enamel matrix secretion and mineralization (Woltgens et al., 1987). During amelogenesis ameloblasts undergo three major stages: secretory, transitional and maturation stages. At the secretory stage, ameloblasts secrete large amounts of enamel matrix proteins forming a scaffold within which HA crystals are formed almost immediately when the enamel matrix is laid down. The first HA crystals that are formed interdigitate with dentin crystals (Arsenault and Robinson, 1989). An important event for the production and organization of the enamel is the development of a short cytoplasmic extension on the secretory end of ameloblasts, the Tomes' processes. Up to 50% of ameloblasts may undergo apoptosis during transitional and maturation stages (Smith and Warshawsky, 1977) and the number of cells of the stratum intermedium and stellate reticulum are significantly reduced by apoptosis during advancing enamel formation (Vaahtokari et al., 1996), preceding total disappearance of these cells upon tooth eruption. Hence, enamel cannot renew itself. TGF-β1

has been suggested to be responsible for the apoptosis observed during the maturation stage (Tsuchiya et al., 2009).

Protein composition and completion of mineralization. - Amelogenins (5-28 kDa) compose over 90% of proteins in the forming enamel matrix (Fincham et al., 1999). Diversity of the amelogenins found in enamel matrix is due to alternative mRNA splicing and proteolysis (Simmer et al., 1994). The remaining 10% of enamel proteins are less abundant, such as ameloblastin and enamelin (Hu et al., 1997; Krebsbach et al., 1996). Amelogenin, ameloblastin and enamelin all belong to the secretory calcium-binding phosphoprotein gene family (Kawasaki and Weiss, 2003) and are controlled by vitamin D (Papagerakis et al., 2002b; 2003).

Amelogenins are bipolar molecules with a hydrophilic carboxyl terminus while the bulk of the molecule is hydrophobic (Fincham et al., 1999). Following secretion, amelogenins assemble to form supramolecular aggregates, nanospheres, which are thought to control crystal growth, morphology and orientation by surrounding crystals along their long axis and preventing crystals from fusing during their formation (Fincham et al., 1994; Yang et al., 2010). Amelogenins are abundant throughout the developing enamel matrix, although uncleaved amelogenin polypeptides concentrate in the newly synthesized outer enamel layer (Aoba et al., 1992; Uchida et al., 1991). *Amelogenin* knockout mice exhibit major structural enamel defects that affect the overall enamel thickness and rod (prism) structure (Gibson et al., 2001).

Members of the nonamelogenin family of enamel proteins are believed to promote and guide the formation of enamel crystals. For example, ameloblastin (45 kDa) is a glycoprotein, which comprises about 5% of enamel proteins, and is mainly expressed by ameloblasts (Krebsbach et al., 1996). In *ameloblastin*-null mutant mice, the dental epithelium differentiates into enamel-secreting ameloblasts, but the cells detach from the matrix at the secretory stage and lose their polarity (Fukumoto et al., 2004). Hence, no structured enamel layer is created in the absence of ameloblastin (Fukumoto et al., 2004). Thus, Fukumoto et al. (2004) suggest that ameloblastin is a key adhesion molecule for ameloblasts and it plays an important role in maintaining the differentiated phenotype of secretory ameloblasts.

Enamelin is the largest enamel protein (porcine enamelin 186 kDa; Hu et al., 1997) and the least abundant (1-5%). It is a glycosylated, phosphorylated protein expressed primarily by ameloblasts (Hu et al., 2003). *Enamelin*-null mutant mice do not make true enamel, and despite the accumulation of a thick layer of enamel proteins in the extracellular space, mineral formation is almost completely absent (Hu et al., 2008). Among a series of enamelin cleavage products a 32-kDa enamelin has been shown to interact with amelogenin and proposed to regulate amelogenin macromolecular self-assembly (Fan et al., 2009).

Once the full thickness of enamel has been deposited, secretory ameloblasts pass through a short transitional stage into the maturation stage (Figure 2). At the end of the transitional stage, ameloblasts deposit a basal lamina, which adheres to the enamel surface, and the ameloblasts attach to it (Moffatt et al., 2006). One component of the basal lamina is amelotin (AMTN) (Moffatt et al., 2006). At the maturation stage ameloblasts are responsible for degradation of enamel matrix proteins resulting in final mineralization of the enamel as preexisting HA crystals grow in width and thickness. The crystallites thicken until they press against one another (Smith, 1998). During the maturation stage, ameloblasts cycle between smooth and ruffle-ended phases of their apical surfaces. Enamelysin (MMP-20), a calcium-dependent matrix metalloproteinase, is one of the proteinases processing amelogenins during

enamel mineralization at the secretory and early maturation stages (Caterina et al., 2002; Hu et al., 2002). MMP-20 has also been shown to cleave ameloblastin *in vitro* (Iwata et al., 2007) and enamelin is rapidly cleaved following its secretion, probably by MMP-20 (Hu et al., 2003). Another enzyme, kallikrein 4 (KLK4), a serine proteinase, degrades enamel proteins particularly during the maturation stage facilitating the removal of organic material from the enamel (Hu et al., 2002; Simmer et al., 2009). For example, KLK4 further processes the most stable cleavage product of enamelin, the 32-KDa enamelin (Hu et al., 2003). KLK4 is also expressed by odontoblasts (Nagano et al., 2003).

Proper processing of enamel matrix proteins is essential for the removal of organic material during the mineralization and maturation stages of enamel development (Bartlett et al., 2004). Loss of function of the associated proteolytic enzymes produces hypomineralized enamel; *Mmp-20* null mice have a thinner than normal (hypoplastic) enamel layer, which has decreased mineral content, lacks normal rod (prism) structure and tends to delaminate from the underlying dentin (Bartlett et. al, 2004; Caterina et al., 2002). Furthermore, inhibition of MMP activity impairs enamel formation and mineralization in cultured mouse embryonic molars (Bourd-Boittin et al., 2005).

Ocn has also been shown to be expressed by epithelial cells, for example, in the enamel free area of the cusp tips of rodent molars and stratum intermedium-like cells in odontogenic mixed tumors (Bosshardt and Nanci, 1997; Papagerakis et al., 1999). Papagerakis et al. (2002a) detected Ocn protein in human enamel matrix specifically at the maturation stage by immunohistochemistry. Bronckers et al. (1998) observed that the earliest deposits of mineralizing enamel were immunopositive for Ocn and Ortiz-Delgado et al. (2005) showed staining in enameloblasts and enamel in teleost fish. However, the role of Ocn in amelogenesis is unknown.

TNAP is expressed in ameloblasts and it is likely to have a role in enamel formation, too (Hotton et al., 1999). Incisors of *TNAP* mutant mice show hypomineralized enamel (Waymire et al., 1995). Further, increased enamel width has been observed in biglycan null teeth and impaired enamel formation in decorin null teeth (Goldberg et al., 2002), which implies PG action also in amelogenesis. Increased enamel formation in the biglycan null teeth results most probably from enhanced amelogenin synthesis whereas delayed enamel formation in the decorin null teeth has been suggested to be an indirect consequence of dentin hypomineralization (Goldberg et al., 2005). Cell-cell and cell-matrix adhesions are important as well for the proper enamel formation. *Nectin-1*;*nectin-3* compound mutant mice have severely reduced expression of cell adhesion proteins in the enamel organ epithelium and impaired desmosomal junction formation between the stratum intermedium layer and maturation stage ameloblasts, which seems to lead to retarded enamel maturation (Yoshida et al., 2010).

1.1.3.3. Root cementum

Root cementum is a mineralized tissue which covers dentin on the root surface. Cementum anchors the collagen fibers that connect the root to the alveolar bone through the periodontal ligament. On the cervical half of the root there is a thin acellular extrinsic fiber cementum (primary cementum or acellular cementum), for which the degree of mineralization is about 45-60%, and on the apical half of the root there is a thick cellular intrinsic fiber cementum (secondary cementum or cellular cementum). A characteristic feature of cellular cementum is the presence of cementoblasts (cementocytes) entrapped in lacunae within the matrix.

Cellular cementum is not as well mineralized as acellular cementum, and can be reactively thickened.

As in the case of dentin and bone, type I collagen is the predominant organic component of cementum constituting up to 90% of the organic matrix. Cementum also contains other collagen types and many noncollagenous proteins (NCPs) (Bosshardt, 2005). Cementum NCPs include BSP, DMP-1, DSPP, fibronectin, osteocalcin, osteonectin, osteopontin, tenascin, proteoglycans, proteolipids, and several growth factors (Nanci and Bosshardt, 2006). Furthermore, enamel proteins have also been detected in cementum (Nunez et al., 2010).

Both acellular and cellular cementum formation is initiated with the deposition of collagen fibrils by cementoblasts within the unmineralized root dentin surface. Cementoblasts also secrete NCPs, which regulate mineralization of the cementum. Mineralization of root dentin starts internally and reaches the dentin surface after the collagen fibrils of dentin and cementum have blended together. Mineralization then spreads across the dentin-cementum junction into the cementum. Collagen fibrils of the outermost layer of acellular cementum are formed by periodontal ligament fibroblasts, therefore it is also referred to as extrinsic fiber cementum. There is no morphologically distinct unmineralized layer on the surface of acellular cementum, whereas during the formation of cellular cementum, a layer of unmineralized matrix, cementoid, is formed and then mineralized. When cementogenesis of cellular cementum continues, some cementoblasts become trapped in the matrix, thus it is named cellular cementum. (Nanci and Bosshardt, 2006.)

A prominent manifestation of human hypophosphatasia, resulting from defects in tissue non-specific alkaline phosphatase (TNAP), is the early loss of teeth, which most likely traces back to cementum agenesis (Chapple, 1993; van den Bos et al., 2005). This is suggestive of an essential role of TNAP in cementogenesis. Accordingly, in TNAP-deficient mice acellular cementum formation is defective (Beertsen et al., 1999).

1.2. Disturbed dental hard tissue formation

1.2.1. Background and clinical aspects

Disturbances in tooth development may be attributed to genetic or environmental reasons. However, the majority of these defects are etiologically idiopathic. Environmental disturbances can further be divided to systemic (e.g. caused by certain drugs, environmental toxicants and fluoride) and local (e.g.caused by infection and irradiation).

Developmental disturbances impacting on the enamel are clinically grouped into demarcated and diffuse hypomineralizations, for which the enamel is improperly mineralized and seems whiter or yellower than normal, and into hypoplasias, for which the amount of enamel matrix is reduced. Any interference to amelogenesis at the secretory stage is associated with thin (hypoplastic) enamel. Disturbances during the maturation stage result in soft enamel of normal thickness.

Knowledge of the etiology of hereditary dentin and enamel defects in mice and humans may help reveal mechanisms of impaired dental hard tissue formation caused by environmental toxicants. On the other hand, *in vitro* animal studies on the effects of environmental toxicants

on developing teeth can increase the understanding of mechanisms of aberrant human tooth development, especially those due to environmental impact. Further, experimental studies may help assess human risk for developmental dental disturbances of environmental background.

1.2.2. Inherited defects

1.2.2.1. Dentin defects

The two most common hereditary diseases affecting dentin are dentinogenesis imperfecta (DGI, types I-III) and dentin dysplasia (DD, types I and II). Thus far, more than 30 different mutations in the human *DSPP* gene have been associated with DGI types II and III and DD type II (Nieminen et al., 2010; McKnight et al., 2008). DGI type I, included in the original classification of heritable human dentin defects (Shields et al., 1973), is the dental manifestation of a heterogeneous, generalized connective tissue disease, osteogenesis imperfecta (OI), which results from various mutations in the genes encoding type I collagen chains. Patients with DGI typically have amber-brown, opalescent teeth with soft dentin and although the enamel is structurally normal, it is often broken off. In DD type I, all teeth are normal in shape and color but have short roots contributing to early exfoliation. Periapical radiolucencies are frequently detected. Primary teeth in DD type II are phenotypically similar to DGI, but permanent teeth appear normal or show only mild discoloration.

1.2.2.2. Enamel defects

Development of the dental enamel is exclusively impaired in clinically and genetically heterogeneous disorders with at least 14 different subclasses, called amelogenesis imperfecta (AI) (Witkop, 1988). AI patients have abnormalities primarily in the amount, composition, and/or structure of the enamel, and accordingly, the disorder is classified as hypoplastic, hypomature or hypocalcified (Witkop, 1988). Mutations in five genes, amelogenin (*AMELX*), enamelin (*ENAM*), family with sequence similarity, member H (*FAM83H*), *KLK4*, and *MMP-20*, have been reported to cause AI in humans, and the mode of inheritance is autosomal-dominant, autosomal-recessive, or X-linked (Hart and Hart, 2009). Mutations in *AMELX* and *ENAM* genes cause varied degrees of hypomineralization and/or hypoplasia ranging from localized pitting to generalized thinning of the enamel (Kim et al., 2005; Rajpar et al., 2001; Wright et al., 2003; 2009b). Impaired function of MMP-20, or of KLK4, results in hypomaturation type AI, where enamel is of normal thickness but with deficient mineralization (Hart et al., 2004; Wright et al., 2009b). Mutations in *FAM83H* are associated with hypocalcified AI and markedly decreased mineral content in the enamel (Wright et al., 2009a).

Enamel defects are also associated with various syndromes and diseases. For example, the phenotypes of tricho-dento-osseous syndrome, vitamin D-dependent and vitamin D-resistant rickets, and APECED, that is autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy, involve enamel hypoplasia (Bailleul-Forestier et al., 2008). Patients with coeliac disease have enamel defects ranging from defect in color to severe hypoplasias, and patients with epidermolysis bullosa suffer from variable degrees of hypoplasia (Aine, 1986; Wright et al., 1993).

1.2.3. Defects caused by systemic environmental factors

Under the designation of environmental dental defects, only changes in the enamel are conventionally described although there would be changes in the dentin too. This is because enamel defects are easier to identify clinically.

Susceptibility of the developing enamel to hypomineralization defects of environmental origin is highest at the transitional and early maturation stages of amelogenesis (Suga, 1989). It is well known that excess fluoride intake during these stages causes fluorosis, a specific type of enamel hypomineralization. At lower doses, fluoride is the most important caries-preventive agent in dentistry. Exposure to environmental organic toxicants, such as polychlorinated dibenzo-*p*-dioxins and dibenzofurans, has also been connected with enamel defects in humans.

The stage of development of the dentition is dependent on the age of the child and therefore, susceptibility of different teeth to developmental disturbances at different times varies. Development of the first permanent molars and incisors begins at the fourth gestational month and hard tissue formation in them starts around or soon after birth. Enamel formation in the upper first incisors has been completed by the end of the fifth year of life and in the first molars at about three years (Reid and Dean, 2006). Accordingly, human permanent incisors and first molars are at greatest risk for defects caused by systemic environmental factors up to the first years of life (Evans and Darvell, 1995; Moorrees et al., 1963).

Demarcated enamel opacities in 1-4 permanent first molars and frequently incisors is a condition known as Molar-Incisor Hypomineralization (MIH). Besides enamel hypomineralization, interglobular dentin and an increased proportional amount of organic matter in dentin under the affected enamel have been observed (Heijs et al., 2007). The condition is fairly common in different child populations: its prevalence in European countries varies between 3.6-25% (Weerheijm and Mejàre, 2003). Etiology of MIH is largely unclear. However, several factors have been suggested, such as prenatal, perinatal or postnatal medical problems (Fredén and Gronvik, 1980; Jälevik et al., 2001; Lygidakis et al., 2008), use of antibiotics (Laisi et al., 2009), and exposure to dioxins and polychlorinated biphenyls in early childhood (Alaluusua et al., 1996; Jan and Vrbic, 2000).

1.3. Environmental toxicants

1.3.1. Dioxins and dioxin-like compounds

1.3.1.1. Nature and human exposure

Polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and polychlorinated biphenyls (PCBs) are collectively called dioxins and dioxin-like compounds, although the term “dioxins” strictly refers only to PCDDs (Van den Berg et al., 2006). The most toxic and widely studied of this general class of compounds is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, Figure 3), which is often called simply “dioxin”, and which represents the reference compound for this class of compounds.

PCDDs are organic compounds, in which two chlorinated benzene rings are joined by two oxygen bridges. They are produced when organic material containing chlorine is burned, for

example by combustion of municipal waste and in chemical manufacturing. PCDDs are ubiquitous in the environment and liposoluble, thus they accumulate in fat and enrich in food chains. Polychlorinated biphenyls (PCBs) are structurally and conformationally similar to PCDDs. Their chemical structure contains 1 to 10 chlorine atoms attached to a biphenyl, which is a molecule composed of two benzene rings joined by a carbon-carbon bond. PCDDs can be generated through partial oxidation of PCBs. The chemical structure of PCDFs resembles the structure of PCDDs, but benzene rings are fused to one furan ring in the middle. In the following sections, I will mainly focus on the most toxic PCDD, the TCDD.

Humans are exposed to PCDDs, PCDFs and PCBs mainly *via* food, especially milk and dairy products, and meat and fish (Kiviranta et al., 2001). In infancy, children can be exposed to these compounds mainly *via* breast-feeding (Vartiainen et al., 1997). An infant can get even 25% of the mother's dioxin load *via* lactation and the accumulation of dioxins and dioxin-like compounds in fat may prolong the duration of their action (Vartiainen et al., 1997). However, concentrations of dioxins and dioxin-like compounds in mother's milk have been decreasing since the 1970s (Kiviranta, 2005; van Leeuwen and Malisch, 2002; Wilhelm et al., 2007). In human adults, most of TCDD is stored in the adipose tissue and has a half-life of approximately 7 years (Pirkle et al., 1989).

1.3.1.2. Mechanisms of action

The majority of adverse effects of TCDD are mediated by the aryl hydrocarbon receptor (AhR) (Gonzalez and Fernandez-Salguero, 1998), which, after binding to TCDD translocates to the nucleus, dimerizes with the aryl hydrocarbon receptor nuclear translocator (ARNT) and binds to DNA. This initiates the transcription of xenobiotic-metabolizing enzymes such as cytochrome P450 1A1 (CYP1A1), which is known to be the primary target for TCDD-inducible gene expression (Whitlock, 1999). There are also several other AhR-dependent ARNT-independent, non-genomic signaling pathways, through which TCDD has been suggested to act (Marlowe and Puga, 2005). AhR-independent mechanisms of TCDD actions have been studied in AhR-deficient transgenic mice (Fernandez-Salguero et al., 1996). AhR-deficient mice are highly resistant to TCDD-induced toxicity (Fernandez-Salguero et al., 1996). All in all, the understanding of the connections between TCDD as a ligand, signaling mechanism and a certain toxic outcome remains minimal.

1.3.1.3. General effects

Humans. **-** The majority of effects on humans have been reported after occupational or accidental exposure to TCDD. Although studies on humans have revealed a wide spectrum of effects of TCDD (USEPA), the most consistently recognized effect of exposure to TCDD-contaminated substances is chloracne. It is a persistent acneiform condition occurring subsequent to substantial acute and chronic exposure to a variety of halogenated aromatic hydrocarbons (Caramaschi et al., 1981; Tindall, 1985). Elevated levels of serum gamma glutamyl transferase (GGT) have also been observed after TCDD exposure (May, 1982; Mocarelli et al., 1986; Moses et al., 1984). Increased levels of GGT may suggest activity such as cholestases, liver regeneration, or drug or xenobiotic metabolism. In addition, the risk of abnormally low testosterone was two to four times higher in exposed chemical production workers than in unexposed referents (Egeland et al., 1994). Dioxins and dioxin-like compounds are also suggested to increase the prevalence of orofacial clefts (ten Tusscher et al., 2000). The International Agency for Research on Cancer (IARC) graded TCDD as a human carcinogen in 1997.

Animals. **-** Dioxins and dioxin-like compounds, especially TCDD, at relatively high doses cause lethality, wasting, lymphoid, thymic, splenic and gonadal atrophy, chloracne and other changes in the skin, hepatotoxicity, altered lipid metabolism, adult neurotoxicity, cardiotoxicity, and disturbances in the endocrine system. However, TCDD is developmentally toxic, for example, to immune, nervous, and reproductive systems at concentrations even close to the prevailing levels, and causes fetotoxicity, growth retardation and thymic and splenic atrophy at doses below those, which are toxic to the mother. TCDD also induces orofacial clefts and hydronephrosis. (Birnbaum and Tuomisto, 2000.)

1.3.1.4. Effects on tooth development

Humans. **-** Current knowledge about the effects of dioxins and dioxin-like compounds on human tooth development is mainly based on accidents, in which loads of these harmful toxicants have been released into the environment. For example, exposure of children to TCDD after the dioxin accident in Seveso, Italy, in 1976, has been found to correlate with the occurrence of developmental enamel defects (Alaluusua et al., 2004). Accordingly, enamel defects were detected more often in Slovakian children exposed to high concentrations of PCB than in children exposed to lower concentrations (Jan et al., 2007). Yet, prevailing concentrations in the environment may be sufficiently high to impair tooth development: MIH was associated with PCDD/PCDF exposure of the child *via* mother's milk in a Finnish study (Alaluusua et al., 1996) but not in another study performed about 10 years later with lower exposure levels (Laisi et al., 2008).

Animals. **-** PCDD/PCDF exposure of bank voles in a contaminated sawmill area caused reduced size of their third molars (Murtomaa et al., 2007). Further, experimental studies on rat and mouse teeth *in vivo* and in organ culture have confirmed developmental dental toxicity of TCDD. The ultimate effect of exposure of cultured mouse embryonic mandibular molars at the initiation stage of development is arrest of tooth development by apoptosis (Partanen et al., 2004). TCDD enhances apoptotic death of those dental epithelial cells that are predetermined to die apoptotically, that is the cells of the OEE, stellate reticulum, stratum intermedium, IEE and dental lamina (Partanen et al., 2004). Furthermore, rat pups exposed to TCDD also *in utero* and through lactation, within a limited time frame, display a failure of tooth development (Kattainen et al., 2001; Lukinmaa et al., 2001; Miettinen et al., 2002). *In vitro* and *in vivo* exposure at the bud, cap and bell stages leads to disturbed cuspal morphogenesis and diminished tooth size (Kattainen et al., 2001; Lukinmaa et al., 2001; Miettinen et al., 2002; Partanen et al., 1998; 2004). At later, formative stages, mineralization of dentin matrix and deposition of enamel matrix were impaired *in vitro* (Partanen et al., 1998). TCDD exposure of rat pups also *via* lactation retarded deposition of dentin and enamel matrices, impaired dentin mineralization and delayed degradation of enamel matrix during enamel maturation in developing molar teeth. Root development was also arrested (Gao et al., 2004; Lukinmaa et al., 2001). The effects of TCDD substantially depend both on the dose or the concentration and the developmental stage of the tooth.

AhR and ARNT are coexpressed in developing mouse molars during early stages of development and mineralization (Sahlberg et al., 2002), and CYP1A1 is expressed in rat molar ameloblasts and odontoblasts at the mineralization stage (Gao et al., 2004). In a previous microarray study, expressions of *Cyp1a1* and *Cyp1b1* were highly upregulated in mouse E14 mandibular molar tooth germs exposed to TCDD for 24 hours (Sahlberg et al., 2007). Metabolism of dioxins and dioxin-like compounds by CYP1A1 and CYP1B1 can result in the generation of mutagenic metabolites and free radicals (Nebert et al., 2000).

These may disturb the normal, intrinsic cell functions, which may lead to apoptosis and retarded tooth development and matrix formation. Changes in the expression of genes involved in tooth development were rather small due to TCDD exposure (Sahlberg et al., 2007). This implies that the effect of TCDD on dental tissues is indirect and that the adverse effects on deposition and mineralization of dentin and enamel, caused by TCDD, may result from modified expression of several developmentally regulated genes (Sahlberg et al., 2007). On the other hand, TCDD decreased *Dspp* gene expression in cultured mouse E18 mandibular molars exposed for at least 3 days (Kiukkonen et al., 2006). TCDD did not affect *Mmp-20* expression in ameloblasts but in odontoblasts the expression was slightly decreased (Kiukkonen et al., 2006). Thus, target genes of TCDD action may be temporo-spatially determined.

For the adverse effects of TCDD on early dental hard tissue formation to become evident in cultured mouse embryonic tooth germs, epidermal growth factor receptor (EGFR) expression is required (Partanen et al., 1998). EGFR expression is strong in IEE but decreases or is not detected in preameloblasts or secretory ameloblasts (Davideau et al., 1995; Heikinheimo et al., 1993; Partanen and Thesleff, 1987). In contrast, Fujiwara et al. (2009) observed expression in secretory ameloblasts. During the maturation stage, the expression in ameloblasts increases again (Davideau et al., 1995). EGFR is also expressed in preodontoblasts and odontoblasts (Davideau et al., 1995; Fujiwara et al., 2009). The results suggest that these cells may be targets of TCDD action.

1.3.2. Non-halogenated polycyclic aromatic hydrocarbons

1.3.2.1. Nature, human exposure, general effects and mechanism of action

The chemical structure of non-halogenated polycyclic aromatic hydrocarbons (PAHs) contains three or more fused aromatic rings but not anionic halogens as seen in the chemical structure of dioxins and dioxin-like compounds (Figure 3). PAHs are ubiquitous, toxic, environmental contaminants that are formed by incomplete combustion and carbonization processes of organic matter. Apart from some natural sources like smoke from forest fires and volcano eruptions, they are present in industry- and traffic-derived exhaust fumes, industrial byproducts, and food. A number of different PAHs, such as benzo[a]pyrene (BP) and benz[a]anthracene, are one of the main toxic components of tobacco smoke (Hoffmann et al., 2001). Among nonsmokers, the principal route of PAH exposure is through the diet (Ramesh et al., 2004). The range of the dietary load of PAHs has been reported to be 0.02–28 μg/person/day (Ramesh et al., 2004). Tobacco smoke increases the daily PAH dose in an average of 0.1–0.25 μ g per a nonfilter cigarette (Hoffman and Hecht, 1990). Hence, an average total PAH yield may be as high as 30 μg per day for heavy smokers (Menzie et al., 1992).

The placenta protects the fetus by filtering noxious compounds from the maternal blood. Even so, the amount of BP metabolites bound to DNA as measured from placenta and umbilical cord blood was higher among smoking mothers than their non-smoking counterparts (Arnould et al., 1997). Furthermore, the breast milk of smoking mothers contains substantially higher concentrations of PAHs than milk of non-smoking mothers (Zanieri et al., 2007). Children of heavy smokers living in urban areas may get BP *via* breast milk up to 1000 times more than the acceptable daily intake for drinking water established by the European commission (Zanieri et al., 2007).

As demonstrated in animal experiments *in vivo* and in tissue and cell cultures *in vitro*, PAHs are cytotoxic and genotoxic, and therefore cause carcinogenicity, and hemato-, cardio-, neuro-, immuno-, renal-, reproductive-, and developmental toxicities (Ramesh et al., 2004). Furthermore, the IARC has graded benz[a]anthracene as possibly carcinogenic to humans in 2010 and is currently upgrading BP as a human carcinogen.

The adverse effects of PAHs, like those of dioxin, are thought to be mediated by the Ah receptor; the presence of the cytosolic AhR has been shown to be required for the toxicity of PAH compounds to become evident (Gonzalez and Fernandez-Salguero, 1998; Mann et al., 1999; Matikainen et al., 2001). Binding of PAHs to AhR leads to transcription of certain genes such as *CYP1A1* and *CYP1B1* (Shimada et al., 2003; Uno et al., 2004). CYP1A1 and CYP1B1 metabolize PAH compounds to active metabolites that are for the most part responsible for the toxic effects of PAHs (Gonzales, 2001).

1.3.2.2. Effects on tooth development

Clinical studies suggest that maternal smoking during pregnancy can reduce the crown size of the child's deciduous and permanent teeth (Heikkinen et al., 1992; 1994a; 1994b). Accelerated clinical eruption and thinning of permanent incisors have also been reported (Heikkinen et al., 1995; 1997). Delayed maturation of permanent teeth, that is, delayed dental age compared with chronological age has been observed in children whose parents smoke (Kieser et al., 1996). PAH compounds are suggested in part to be responsible for these detrimental effects. On the other hand, the frequency of enamel defects in the permanent first molars was not found to be increased in children whose mothers had smoked during the last 12 months before delivery (Alaluusua et al., 1996).

To the best of my knowledge, no *in vivo* or *in vitro* studies on effects of non-halogenated polycyclic aromatic hydrocarbons on tooth development have been conducted before the present thesis project.

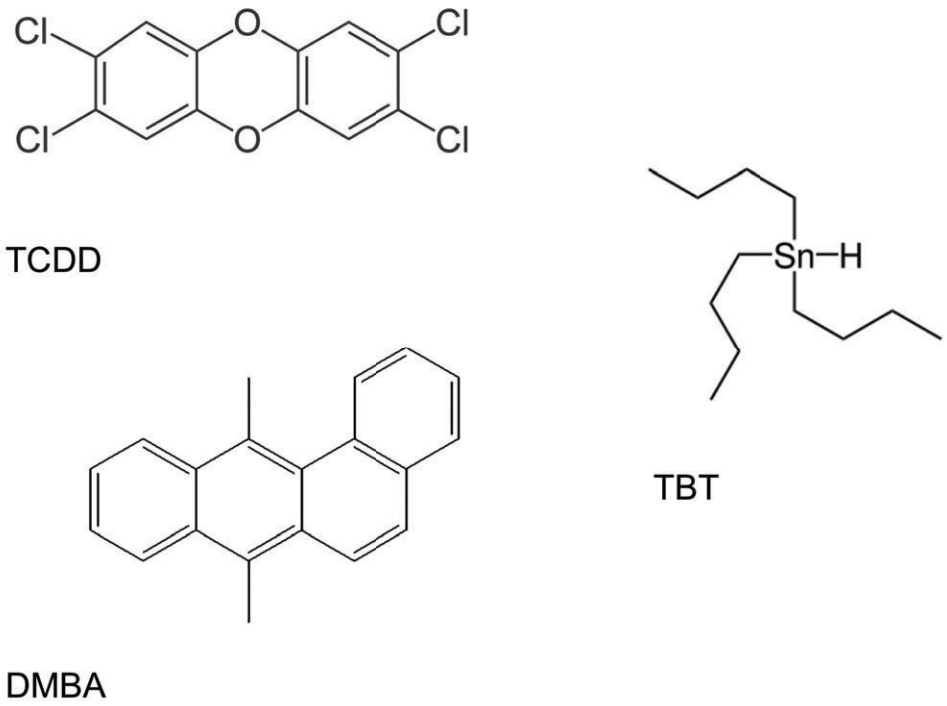

Figure 3. The structural formulas of TCDD, DMBA and TBT.

1.3.3. Organic tin compounds

1.3.3.1. Nature, human exposure and general effects

Organic tin compounds (OTCs) are composed of tin, which binds from one to four hydrocarbon groups (mono-, di-, tri-, and tetrasubstituted) (Figure 3). Organotins are most widely used for stabilization of polyvinyl chloride as mono- or di-substitutes (Risk and Policy Analysts limited [RPA], 2005). A tri-substituted organotin, tributyltin (TBT), was widely used as an antifouling agent in marine paints until it was observed to accumulate in aquatic animals and cause severe damage to the aquatic ecosystem (Harino et al., 2000; Strand and Jacobsen, 2005). Despite restrictions in the use of TBT since the 1980's, high concentrations have been found in coastal areas of harbors and dockyards, heavily used water routes and dredge spoils sites (Antizar-Ladislao, 2008). The International Maritime Organization has prohibited the use of TBT as an antifouling biocide since the year 2008.

The intake of TBT has been assessed to cause risks of concern for humans (RPA, 2005). Humans are exposed to TBT mainly *via* seafood in the diet (RPA, 2005). The average intake of TBT by humans from market-bought seafood has been estimated to vary worldwide between 0.18 and 2.6 μg/day/person (Keithly et al., 1999). However, humans can be exposed to variety of organotins through a wide range of products and other sources. The cumulative intake may in fact exceed the tolerable daily intake (TDI) (RPA, 2005). Recommendation of the European Food Safety Agency for the highest allowed daily intake of organotins is 0.25 μg/kg body weight (European Food Safety Authority, 2004). Children are estimated to be particularly at risk of being exposed to organotin levels exceeding the TDI values (RPA, 2005). This causes great concern, because TBT has been found to cause developmental defects in mammals.

There are no epidemiologic data on human health effects of chronic low level exposure to OTCs but some case reports describe problems after acute exposure to TBT (Grace et al., 1991; Wax and Dockstader, 1995). The toxicity of TBT at low concentrations has been demonstrated in mammals, fish and mollusks. TBT has been shown to cause imposex, that is, development of additional male sex organs in female snails, oysters and fish, which leads to problems in reproduction (Horiguchi et al., 1998; Shimasaki et al., 2003; Smith, 1981). Cleft palate and aberrant gonadal development have been observed in rodents exposed to TBT chloride (TBTCl) *in utero* (Ema et al., 1997; Kishta et al., 2007). Experimental studies imply that organotins can transfer to offspring through the placenta and lactation (Kimura et al., 2005; Noland et al., 1983).

Previous studies show that TBT and another tri-substituted organotin, triphenyltin, interfere with biomineralization in different species (Adeeko et al., 2003; Chagot et al., 1990; Suzuki et al., 2006; Tsukamoto et al., 2004). Defective shell calcification has been reported in oysters (Alzieu, 2000), whilst rodent embryos exposed *in utero* showed delayed ossification of the skeleton (Adeeko et al., 2003; Sarpa et al., 2007; Tsukamoto et al., 2004). *In vitro* TBT impairs the formation of mineralized nodules and deposition of calcium by cultured rat calvarial osteoblasts (Tsukamoto et al., 2004), and decreases osteoblastic activity in teleost scale (Suzuki et al., 2006). Furthermore, Tsukamoto et al. (2004) observed that TBT decreases the expression of genes encoding tissue non-specific alkaline phosphatase (*Alpl*) and osteocalcin (*Ocn*), both associated with mineralization, and inhibits TNAP activity in rat calvarial osteoblast-like cells. Tsukamoto et al. (2004) concluded that TBT could impair differentiation of osteoblasts and that the reduced *Alpl* and *Ocn* expressions are a marker of this effect.

The exact mechanism by which TBT causes adverse effects remains to be elucidated. However, TBT is known to inhibit aromatase, the cytochrome-P450 19a1, which converts testosterone into estradiol (Cooke, 2002; Heidrich et al., 2001). Estradiol, on the other hand, contributes to osteoblast differentiation by enhancing BMP-induced Runx2 and osteocalcin expressions (Matsumoto et al., 2010; Okazaki et al., 2002). Like ostaocalcin, Runx2 also plays a role in tooth development. It has been shown *in vitro* to up-regulate *Dspp* gene expression in mouse preodontoblast-like cells and to reduce the expression in odontoblast-like cells (Chen et al., 2005).

1.3.3.2. Effects on tooth development

To the best of my knowledge, no *in vivo* nor *in vitro* studies on the effects of organic tin compounds on tooth development have been conducted before the present thesis project. There are also no findings in humans.

1.4. Fluoride

1.4.1. Sources and toxicity

Fluoride is a monovalent ion and due to its abundant existence in the nature, fluoride is found at low concentrations in drinking water and foods. Furthermore, it is the most important caries-preventive agent in dentistry. Infants acquire fluoride mainly from infant formulas diluted to water containing fluoride and by early use of fluoride toothpaste (Osuji et al.,

1988). Fluoride content in mother's milk is rather low irrespective of the concentration in drinking water (Chowdhury et al., 1990; Chuckpaiwong et al., 2000).

Fluoride is acutely toxic at high concentrations and can even be lethal. Acute toxicity can occur due to a single ingestion of a large amount of fluoride. However, ingestion of an acute fatal dose is very rare. The probable toxic dose of fluoride has been set to 5.0 mg F per kg body weight. Symptoms of acute toxicity occur rapidly and include diffuse abdominal pain, diarrhea, vomiting, excess salivation, and thirst.

Chronic exposure to excessive fluoride is known to cause dental and skeletal fluorosis in humans. Skeletal fluorosis is a rare chronic metabolic bone and joint disease, which is associated with chronic joint pain, immobilization of joints, and combination of osteosclerosis, osteomalacia and osteoporosis of varying degrees (Krishnamachari, 1986). However, fluoride content in drinking water must be over 4 ppm to cause detectable effects (Kaminsky et al., 1990).

The caries-protective effect of fluoride is based principally on the formation of fluoridated hydroxyapatite. Compared to carbonated apatite fluorapatite crystals dissolve at lower pH values (ten Cate, 1999).

1.4.2. Effects on tooth development

Prolonged excessive ingestion of fluoride during dental hard tissue formation causes fluorosis, which is a specific type of enamel hypomineralization. The dose-response relation is clearly linear, and there is no critical threshold for fluoride intake, below which the effect on enamel will not be manifest. Fluoride causes enamel changes ranging from thin, white, opaque lines to entirely chalky white enamel (Thylstrup and Fejerskov, 1978). Histologically, hypomineralization defects are mainly in the subsurface enamel (Fejerskov et al., 1994). Pits and discoloration are post-eruptive damages to the severely porous and hypomineralized outer enamel (Thylstrup and Fejerskov, 1979). The structural arrangements of the enamel crystals in rod and interrod enamel appear normal but there are enhanced intercrystalline spaces (Fejerskov et al., 1974). Dentin in fluorotic human teeth shows hypermineralization (Rojas-Sánchez et al., 2007). The prevalence of dental fluorosis has increased over the years because the mean fluoride intake from all sources has increased since the 1940s (Mascarenhas, 2000).

Typical enamel hypomineralization defects have also been observed in different mouse strains exposed to fluoride (Everett et al., 2002). Moreover, chronic exposure to fluoride *via* drinking water reduced the enamel thickness of rat incisors (Smith et al., 1993). The susceptibility between different strains varies suggesting a genetic influence (Everett et al., 2002). The amount of fluoride incorporated into mineralized tissues in rats was closely related to the serum-fluoride levels (Speirs, 1986). Fluoride has also been shown to impair enamel matrix secretion and mineralization, and dentin mineralization *in vitro* (Bronckers et al., 1984). Bronckers et al. (1984) observed that fluoride accentuates the globular shape of the mineralization front of dentin, suggesting a sparse distribution of mineralization nodules.

Fluorosis is thought to involve defective degradation and removal of amelogenins from the enamel matrix, which is needed to create space for the mineral phase (Den Besten, 1986). The amount or activity of MMP-20 has been shown to decrease after fluoride exposure during enamel maturation (DenBesten et al., 2002). By decreasing MMP-20 activity, fluoride

could also inhibit degradation of dentin sialophosphoprotein and consequently, affect dentin mineralization. Robinson et al. (2004) proposed that enhanced protein interaction with the mineral could be responsible for both protein retention and reduced proteolysis in fluorotic tissue. Bronckers et al. (2009), for one, suggest that fluoride stimulates crystal formation resulting in the generation of excess protons, which leads to pH drop. This could alter the tertiary structure of amelogenin and affect its function. Low pH also potentiates the inhibitory effect of fluoride on MMP-20 (DenBesten et al., 2002). All in all, the primary molecular mechanism behind fluorosis remains to be clarified.

2. AIMS OF THE STUDY

The principal aim of this project was to examine whether, in addition to dioxins and dioxin-like compounds, other organic environmental toxicants, like non-halogenated polycyclic aromatic hydrocarbons and organic tin compounds, have adverse effects on tooth development *in vitro*, especially on the formation and mineralization of dentin and enamel. The second main aim was to investigate *in vitro* if fluoride could intensify the manifestation of detrimental developmental dental effects of dioxin-like compounds.

The specific aims were to:

1. investigate the effects of 7,12-dimethylbenz[a]anthracene (DMBA) on the size and shape, on the morphology of ameloblasts, and on the formation and mineralization of dentin and enamel of cultured mouse embryonic molar teeth.

2. understand the effects of tributyltin (TBT) on the size and shape, and on the formation and mineralization of dentin and enamel of cultured mouse embryonic molar teeth, and to investigate cellular mechanisms of the dental toxicity of TBT.

3. investigate the effect of TBT on the expression of genes associated with mineralization of dental hard tissues: *osteocalcin* (*Ocn*), *alkaline phosphatase* (*Alpl*), *dentin matrix protein 1* (*Dmp1*), *dentin sialophosphoprotein* (*Dspp*) and *matrix metalloproteinase 20* (*Mmp-20*).

4. establish whether the combined effect of sodium fluoride (NaF) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on dental hard tissue formation is potentiative.

3. MATERIALS AND METHODS

3.1. Teeth and organ culture

Mandibular first and second molar tooth germs of NMRI mouse embryos were dissected under a stereomicroscope on embryonic day 18 (E18). The explants were cultured in a Trowell-type organ culture for 3-12 days and exposed to 7,12-dimethylbenz[a]anthracene (DMBA), tributyltin (TBT), and sodium fluoride (NaF) and/or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). The toxicant concentrations utilised are shown in Table 1. Control explants were cultured in the basal medium without toxicants. The explants were cultured in a humidified incubator at 37ºC in an atmosphere of 5% CO_2 in air. The medium was changed every 2-3 days. At every medium change, the growth of the explants was monitored under a stereomicroscope.

Table 1. Concentrations of the studied toxicants

Toxicant	**Concentrations**
DMBA	0.1, 0.5, 1, 2 μM
TBT	0.1, 0.5, 1.0, 2.0 μM
NaF	2.5, 5, 10, 12.5, 15, 20 μM
TCDD	5, 10, 12.5, 15 nM

3.2. Preparation of explants for histological examination

The teeth were fixed with 4% paraformaldehyde (PFA) in PBS at 4ºC overnight. The explants were demineralized with ethylenediaminetetraacetic acid (EDTA) for at least two weeks, dehydrated through an increasing ethanol concentration series, cleared in xylene, embedded in paraffin and serially sectioned at 7 μm.

3.3. Specific methods (I-IV)

Method	**Material**	**Article**
Organ culture	E18 mouse mandibular molar tooth germs	I, II, III, IV
HE-staining (cell and tissue morphology)	Paraffin sections	I, II, IV
Stereomicroscopic examination (tissue morphology)	Whole explants	I, II, IV
BrdU-staining (cell proliferation)	Paraffin sections	II, IV
TUNEL-staining (apoptosis)	Paraffin sections	II, IV
Digoxigenin *in situ* hybridization (*Ocn*, *Mmp-20*, *Dspp*)	Paraffin sections	III
QPCR (*Ocn*, *Alpl*, *Dmp1*, *Dspp*, *Mmp-20*)	Total RNA from first molars	III
Immunohistochemistry (amelogenin)	Paraffin sections	IV
Statistical analysis		I, II, IV

4. RESULTS

4.1. The development of mouse mandibular first and second molars *in vitro*

4.1.1. The developmental stages at the start of culture (E18) (I, II, III, IV)

At E18 the first molar is at the late bell stage of morphogenesis and its basic cuspal morphology has been completed (Figure 4). By this time, both odontoblasts and ameloblasts are already postmitotic, differentiation of the dentin forming odontoblasts and of enamel forming ameloblasts has started, and deposition of dentin and enamel is about to begin. During the terminal differentiation, odontoblasts and ameloblasts become columnar and polarized, that is, their nuclei become positioned in the non-secreting ends of the cells. Deposition of dentin, and slightly later of enamel, starts close to the cusp tips and proceeds in coronal and cervical directions along slopes of the cusps. In the present organ culture system, mineralization of dentin first occurs in the medial cusp of the first molar, then in the mesial cusp and shortly later in the distal cusp.

At E18, the second molar is undergoing transition from the cap stage of morphogenesis to the bell stage (Figure 4). In the second molar, mineralization is first visible in the mesial cusp and then in the distal cusp.

4.1.2. Stages of development after culture

4.1.2.1. Stages of development after 5 days of culture (E18+5; stereomicroscopic findings) (IV)

In the first molars, odontoblasts had deposited unmineralized predentin throughout the tooth crown. Mineralization of predentin to dentin had begun on the distal slope of the medial cusp and induced ameloblasts to start enamel formation (Figure 4).

In the second molars, predentin deposition had begun but there were no globular mineral foci visible.

4.1.2.2. Stages of development after 10/12 days of culture (E18+10, E18+12; stereomicroscopic and histological findings) (I, II, IV)

In the first molars cultured for 10 and 12 days, odontoblasts had deposited a thick layer of predentin throughout the crown. The extent of mineralized dentin and enamel varied from one culture experiment to another, ranging from the uniform presence in the mesial cusp to the distal cusp in part of the teeth (Figure 4). Ameloblasts were elongated and polarized and they formed a coherent layer (Figure 4). Ameloblasts in the coronal third to half of the mesial slope of the mesial cusp, covering a thick enamel layer had reached the maturation stage, shortened and lost their columnar shape but were still polarized after having secreted the enamel matrix (Figure 4). Correspondingly, odontoblasts located on the mesial side of the mesial cusp were polarized and elongated but had lost their elongated shape at the cusp tip upon predentin formation (Figure 4).

After 10 or 12 days of culture, predentin formation in the second molars was consistently in progress throughout the crowns. The thickness of predentin varied from one culture to another. Predentin mineralization and enamel formation had begun in part of the teeth (Figure 4). Correspondingly, ameloblasts were mostly polarized and at least mesially and distally fully elongated. Odontoblasts were polarized and columnar throughout the tooth crown.

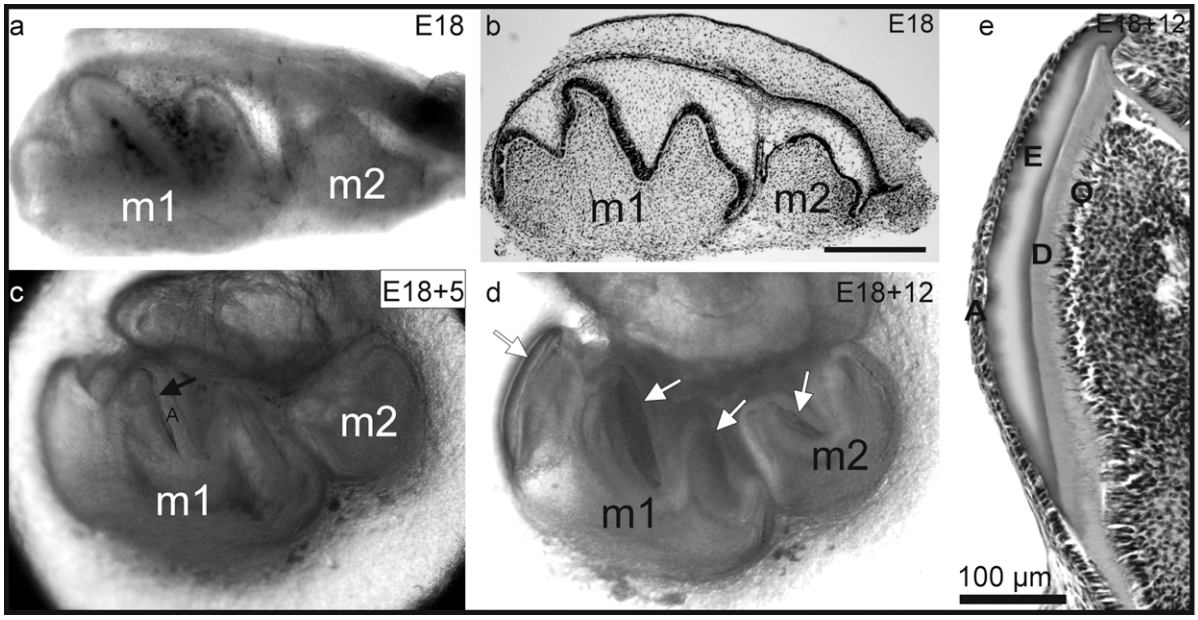

Figure 4. (a, c, d) Stereomicroscopic images of mouse E18 mandibular first (m1) and second (m2) molar tooth germs are shown; (a) at the start of culture, (c) after 5 days of culture, and (d) after 12 days of culture. (b) Histological section of E18 mouse mandibular first and second molars in sagittal plane. (e) Histological section of mesial cusp of E18 mouse mandibular first molar tooth germ after 12 days of culture.

(a, b) At E18 the first molar is at the late bell stage of morphogenesis and its basic cuspal morphology has been completed. The second molar is undergoing transition from the cap stage to the bell stage of morphogenesis. Scale bar in b represents 100 µm. (c) Dentin mineralization (dark grey layer shown by black arrow) has begun in the first molar on the distal slope of the medial cusp, and ameloblasts (A) have started enamel formation. (d) Mineralized dentin and enamel are visible in all three principal cusps of the first molar and in the mesial cusp of the second molar (arrows). (e) Enamel layer (E) facing mineralized dentin (D) extends from the tip of the cusp to the cervical region. Light staining in the central zone of the enamel layer indicates that the maturation phase has started. Ameloblasts (A) in the coronal half of the slope have reached the maturation stage and shortened. Odontoblasts (O) are polarized and elongated but have lost their elongated shape at the cusp tip upon predentin formation.

4.1.3. Apoptosis in developing first and second molars (II, IV)

In the first molars cultured for 7 days, minimal apoptosis was observed in the enamel organ, the dental papilla and the oral epithelium.

In the second molars only few apoptotic ameloblasts were seen.

4.1.4. Cell proliferation in developing first and second molars (II, IV)

In the first molars cultured for 5 and 7 days, proliferating cells were detected in the basal epithelial diaphragm giving rise to Hertwig's epithelial root sheath, and in the juxtaposed mesenchymal cells in the cervical area.

In the second molars cultured for 5 and 7 days, proliferating cells were seen in the basal epithelial diaphragm and in the juxtaposed mesenchymal cells in the cervical area, and in preameloblasts and preodontoblasts. Corresponding to the earlier stage of tooth development, proliferation was more abundant in the second molars than in the first molars.

4.1.5. Amelogenin expression in developing first molar (IV)

In the first molars cultured for 7 days amelogenin was detected by immunohistochemistry in columnar, polarized ameloblasts extending from the cusp tip to the cervical region of the mesial slope of the mesial cusp. Weak staining was present in opposing polarized odontoblasts depositing predentin.

In the first molars cultured for 12 days amelogenin was present in columnar, polarized ameloblasts in the cervical region where enamel matrix secretion was about to start or was in progress, and in the opposing polarized odontoblasts.

4.2. Effects of environmental toxicants and fluoride on cultured mouse mandibular first and second molar development

4.2.1. Effects of 7,12-dimethylbenz[a]anthracene (DMBA) (I)

The effect of DMBA on the first and second molar development was studied after 12 days of culture with special reference to the relationship of DMBA concentration to the morphology of the tooth crown and the dental hard tissue formation.

4.2.1.1. Morphological findings in the first molar

The effect of DMBA on mandibular first molar size was studied by measuring the mesiodistal widths of the tooth crowns from stereomicroscopic images. The size decreased with the increasing DMBA concentration ($p < 0.001$, Pearson's chi-square test, Figure 5). Significant effect between the control and exposed tooth was first seen in explants cultured with 0.5 μM DMBA ($p < 0.02$, Bonferroni's multiple comparison test). DMBA exposure also caused cuspal deformation: cusps were often thin and sharp.

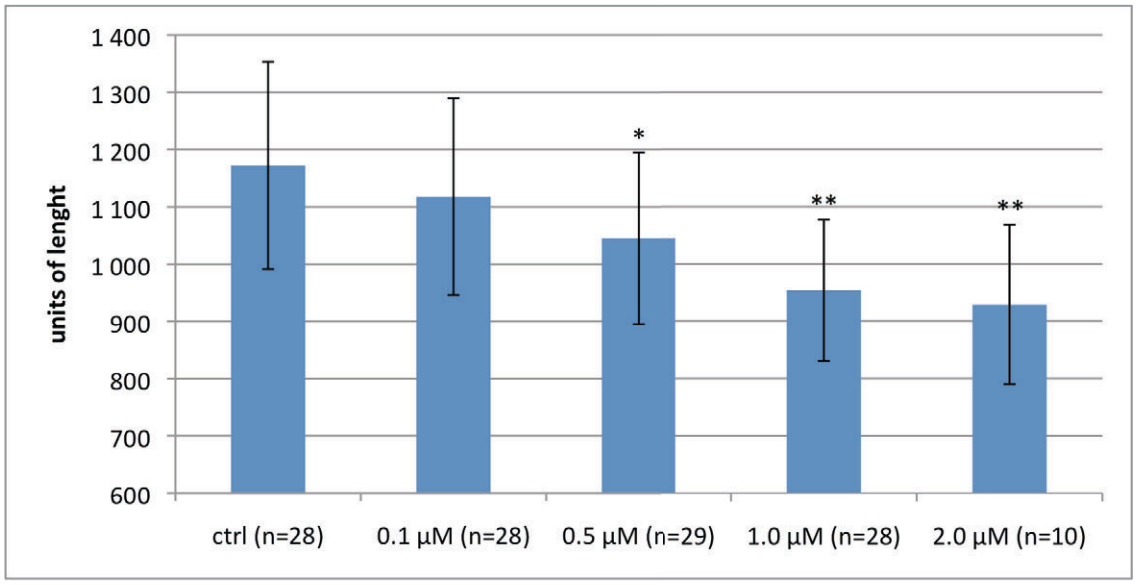

Figure 5. DMBA exposure reduces the mesio-distal width of first molars in a dose-dependent manner ($p < 0.001$, Pearson's chi-square test). The bars show the average tooth size (units of length ±SD) in the control and DMBA exposure groups. Statistically significant difference from the control group is indicated by asterisk; *$p < 0.02$, **$p < 0.0001$, Bonferroni's multiple comparison test.

DMBA disturbed dental hard tissue formation dose-dependently. Predentin was thinner than in controls, mineralization of dentin was retarded and the amount of enamel was reduced or enamel was even lacking.

Elongation of ameloblasts was less obvious than in controls, and part of the ameloblasts was completely nonpolarized as evidenced by the central location of the nuclei in the cytoplasms.

4.2.1.2. Morphological findings in the second molar

Consistent with the changes seen in the DMBA-exposed first molars, the exposed second molars were smaller than controls and their cusps were often thin and sharp. The effects were clear in teeth treated with 0.5 µM DMBA and with higher concentrations. Most teeth exposed to 2 µM DMBA were extremely small and delicate.

Predentin was thinner than in control teeth, and mineralization of dentin or enamel deposition had not started.

Ameloblasts in most teeth had started to elongate, but in some teeth they were largely nonpolarized as evidenced by the centrally located nuclei.

4.2.2. Effects of tributyltin (TBT) (II, III)

The effect of TBT on the first and second molar development was studied after 10 and 12 days of culture with special reference to the relationship of TBT concentration to the morphology of the tooth crown, the dental hard tissue formation and the differentiation and morphology of the dental cells.

4.2.2.1. Morphological findings in the first molar (II)

The effect of TBT on mandibular first molar size was studied by measuring the mesiodistal widths of the tooth crowns from stereomicroscopic images. TBT exposure decreased the size of the tooth with the ascending concentration ($p < 0.001$, Pearson's chi-square test, Figure 6).

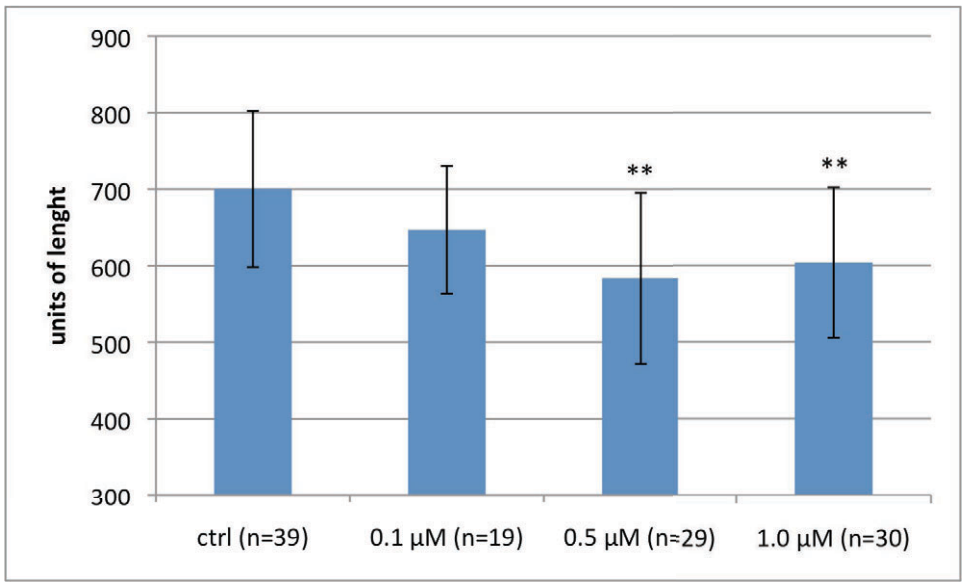

Figure 6. TBT exposure reduces the mesio-distal width of first molars in a dose-dependent manner ($p < 0.001$, Pearson's chi-square test). The bars show the average tooth size (units of length ±SD) in the control and TBT exposure groups. Statistically significant difference from the control group is indicated by asterisk; **$p < 0.0001$, Bonferroni's multiple comparison test.

The effect of TBT on the amount of enamel was estimated from the stereomicroscopic images, and the estimation was confirmed by histological analysis of representative explants. TBT slowed down enamel formation with the ascending concentration ($p < 0.001$, Pearson's chi-square test, Figure 7). TBT retarded dentin mineralization dose-dependently. The effect of TBT on dentin mineralization and enamel formation was clear at the TBT concentration of 0.5 µM and higher. The majority of teeth exposed to 0.5 µM TBT showed clear reduction in dentin mineralization and enamel formation. At the TBT concentration of 1.0 µM, dentin had not started to mineralize and no enamel was visible in the vast majority of teeth (Figure 7).

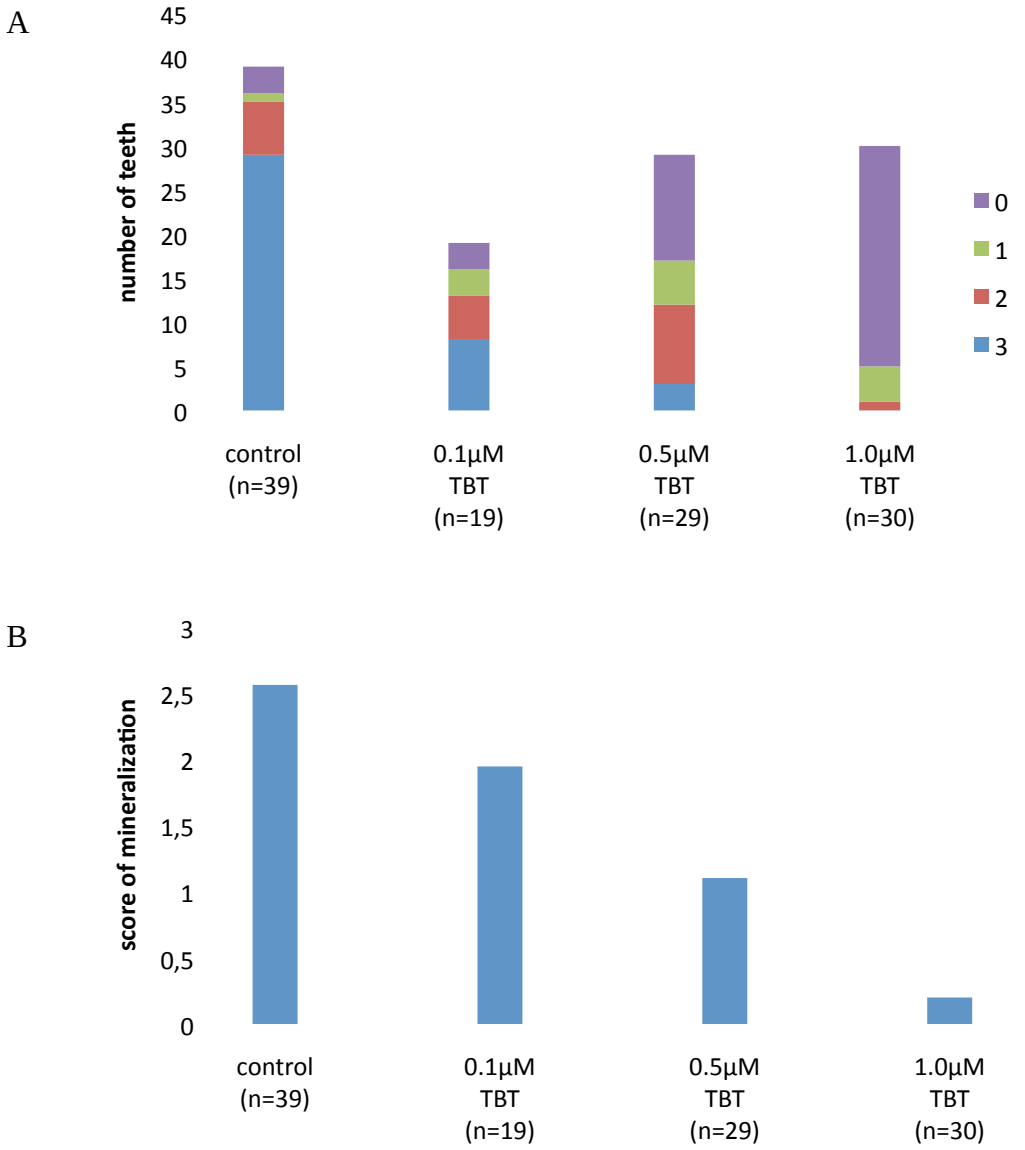

Figure 7. Mineralized dentin and enamel formation in first molars in control and TBT-exposure groups after 12 days of culture. Each first molar was given a score 0–3 depending on the extent and thickness of the enamel on the mesial side of the mesial cusp. Score 3 was given to teeth with a thick and even enamel layer extending from the tip of the mesial cusp at least midway along the mesial side. Score 2 was given to teeth showing clearly thinner or less extended enamel. Score 1 was given if there was only a spot of enamel or mineralized dentin mesially and score 0 if there was no enamel or mineralized dentin at all. In teeth scored 0–2 there was no enamel in the medial and distal cusps. (A) The bars show the distribution of number of scores in each group. (B) The bars show mean values of the given scores of each group. TBT slowed down enamel formation with the ascending concentration ($p < 0.001$, Pearson's chi-square test).

The effect of TBT on predentin formation was less overt. Irrespective of TBT concentration, the thickness of predentin on the mesial side of the mesial cusp in virtually all teeth was comparable to the controls. The proportion of teeth showing reduced predentin thickness in the less advanced medial and distal cusps increased with the concentration of TBT. The reduced thickness of predentin consistently coincided with an altered morphology of the stellate reticulum compartment of the epithelial enamel organ between the medial and distal

cusps. The stellate reticulum cells had lost their normal star-like shape and the loose-textured tissue had become a dense mass of enlarged eosinophilic cells with round, central nuclei, possibly indicating an incipient cell death. Correspondingly, the medial and distal cusps were thin and more or less curly.

The height of ameloblasts decreased with the ascending TBT concentration. Completely nonpolarized ameloblasts with central nuclei were seen especially in the occlusal third of the mesial side of the mesial cusp. Corresponding to the failure of predentin to mineralize, morphological changes in ameloblasts were observed. TBT had no clear effect on odontoblasts on the mesial side of the mesial cusp.

4.2.2.2. Effect on apoptosis and cell proliferation in the first molar (II)

Since cellular effects can be expected to become evident earlier than effects on hard tissue formation, the effects of TBT on apoptosis and cell proliferation were studied from tissue sections of explants cultured for a total of 7 days.

TBT exposure moderately enhanced apoptotic cell death, mainly in epithelial tissues of tooth explants exposed for 5 days to 1.0 μM TBT after two days of culture without TBT. Apoptosis was increased in the enamel organ constituents stellate reticulum, stratum intermedium and ameloblasts; in the basal epithelial diaphragm giving rise to Hertwig's epithelial root sheet; and in the basal layer of the oral epithelium. Apoptosis was concentrated in the medial and distal parts of the first molar but was not seen in the mesial cusp. TBT did not alter the rate of cell proliferation.

4.2.2.3. Morphological findings in the second molar (II)

Consistent with the changes seen in the first molars, TBT affected predentin mineralization and enamel formation in the second molar dose-dependently. There was no mineralized dentin or enamel in any of the teeth exposed to 1.0 and 2.0 μM TBT.

TBT also reduced the thickness of predentin dose-dependently. Where predentin deposition had not started, odontoblasts were nonpolarized. The proportion of teeth showing altered morphology of ameloblasts increased with TBT concentration. Elongation of ameloblasts was impaired in all teeth exposed to 1.0 and 2.0 μM TBT and completely nonpolarized ameloblasts were also visible.

The shape of most teeth exposed to 0.5, 1.0, and 2.0 μM TBT was altered: in sections cusps appeared thin and curly. Cusp tips lacking predentin were disintegrated.

4.2.2.4. Effect on apoptosis and cell proliferation in the second molar (II)

The effects of TBT on apoptosis and cell proliferation were studied from tissue sections of explants cultured for 7 days.

No definite effect on the distribution pattern or frequency of apoptotic cells was observed with 1.0 μM TBT. Apoptosis was mainly seen in ameloblasts and in the epithelial diaphragm.

An increased frequency of BrdU-labeled nuclei on the mesial side of the distal cusp and at the deepest site of the fissure between the cusps was observed with 1.0 μM TBT exposure. Of the

eight teeth exposed to TBT, four showed abundant proliferation in (pre)ameloblasts and stratum intermedium in the region of the whole tooth, in (pre)odontoblasts and in cells of the dental papilla.

4.2.2.5. Effects on gene expression in the first molar (III)

Changes in gene expression during the secretory and mineralization stages of the first molar development after 1.0 μM TBT exposure were studied by real-time QPCR after 3, 5 and 7 days of culture. *In situ* hybridization was used to detect expression patterns of the three genes (*Ocn*, *Mmp-20*, *Dspp*) whose expression showed changed values in QPCR after exposure of tooth explants to TBT. Selected sections of first molars cultured for 5 days were used for hybridization.

After exposure to TBT the expression of *Ocn* was up-regulated 1.5-fold or more (>0.5 in log2-scale) in 70% of the QPCR-assays. After exposure during the whole culture time the relative quantity values of *Ocn* mRNA were higher than the corresponding values of the control sample after 3, 5 and 7 days of culture. When the explants were cultured for the first 2 days without TBT-addition and then exposed for 3 or 5 days (2+3 days and 2+5 days, i.e., total culture time 5 and 7 days, respectively), the results varied.

In control first molars *Ocn* expression was mainly seen in odontoblasts depositing predentin just before the start of mineralization. The expression of *Ocn* decreased in odontoblasts after TBT exposure. In control explants, non-uniform staining was also visible in ameloblasts, stratum intermedium and stellate reticulum cells, especially at the cusp tips. TBT-exposure increased staining in these cells. Since TBT had an opposite effect on *Ocn* expression in odontoblasts and epithelial cells, and QPCR results showed an increased expression in the whole first molar, we performed *in situ* hybridization for first molars also cultured for 3 and 7 (exposed the last 5 days) days to ascertain the result. The result was the same in these slightly earlier and later developmental stages.

After exposure to TBT, the expression of *Mmp-20* was down-regulated 0.7-fold or more (>-0.5 in log2-scale) in 60% of the assays. After exposure during the whole culture time, the relative quantity values of *Mmp-20* mRNA were the same or lower than the corresponding values of the control sample after 5 and 7 days of culture. Expression was down-regulated even when the explants were cultured for the first 2 days without TBT (2+3 days and 2+5 days).

In control first molars *Mmp-20* expression was detected by *in situ* hybridization in mesial ameloblasts and odontoblasts. TBT exposure clearly decreased *Mmp-20* expression in ameloblasts and also in odontoblasts.

The effect of TBT on *Dspp* gene expression varied but the relative quantity values by QPCR were lower than the corresponding values of the control sample after 5 days of culture in 2 of the 3 experiments (explants exposed the whole culture time).

In control first molars *Dspp* expression was abundant in odontoblasts depositing predentin. Consistent with the result from QPCR, TBT exposure decreased *Dspp* expression in *in situ* hybridization.

TBT exposure had no clear effect on *Alpl* and *Dmp1* expressions in QPCR.

4.2.3. Effects of sodium fluoride (NaF) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (IV)

To investigate if simultaneous exposure to NaF and TCDD has a potentiative effect on developing dental hard tissues *in vitro*, mandibular first and second molar tooth germs of E18 mouse embryos were cultured in the presence of each agent alone and together for 5-12 days. Attention was paid to possible changes in cell and tissue morphology, to the expression of amelogenin by ameloblasts and odontoblasts, to apoptosis, and to cell proliferation.

Mesial cusps of first molars cultured for 7 or 12 days were analyzed for amelogenin expression by immunohistochemistry. TUNEL-stained tissue sections of first and second molar explants cultured for 7 days were investigated for apoptosis and BrdU-labeled tissue sections of tooth explants cultured for 5 days for cell proliferation. The stainings were quantified visually under a light microscope.

4.2.3.1. Stereomicroscopic and histological findings

Morphological changes were studied from the whole tooth photographs and histological tissue sections.

Stereomicroscopic findings in the first molar after 5 days of culture. - Simultaneous exposure of first molars to NaF and TCDD at concentrations of 15 μM and 10 nM respectively, which alone had no or a barely detectable effect, impaired predentin mineralization. Changing of the concentrations to 10 μ M NaF and 15 nM TCDD had no effect on the result. NaF and TCDD alone at higher concentrations disturbed predentin mineralization.

Stereomicroscopic findings in the first and second molars after 12 days of culture. - The amount of mineralized dentin and enamel in the first and second molars exposed to 15 μM NaF alone or 10 nM TCDD alone was comparable to controls in the majority of explants. There was no statistically significant difference in the amount of mineralized dentin and enamel ($P > 0.05$, Mann-Whitney test) between control and NaF or TCDD exposure groups in any first molar cusps (Figure 8). However, in the NaF-exposed first molars, the mineralization front of dentin was particularly globular.

In the vast majority of explants exposed to 15 μM NaF and 10 nM TCDD simultaneously, the amount of mineralized dentin and enamel were clearly reduced in the first molars (Figure 8). The differences in the amount of hard tissue between control and NaF+TCDD, between NaF and NaF+TCDD and between TCDD and NaF+TCDD group were statistically significant in the mesial cusp of the first molars ($P < 0.01$, Mann-Whitney test). The difference in the amount of hard tissue between control and NaF+TCDD group was statistically significant in the distal cusp of the first molars ($P < 0.01$, Mann-Whitney test) but non-significant between NaF and NaF+TCDD or between TCDD and NaF+TCDD groups ($P > 0.05$, Mann-Whitney test). The amount of hard tissue was reduced in the medial cusp after simultaneous exposure to NaF and TCDD but the differences were not statistically significant between any of the groups. NaF and TCDD alone at higher concentrations impaired or prevented dentin mineralization and enamel formation.

Dentin mineralization and enamel formation in the second molars were also retarded.

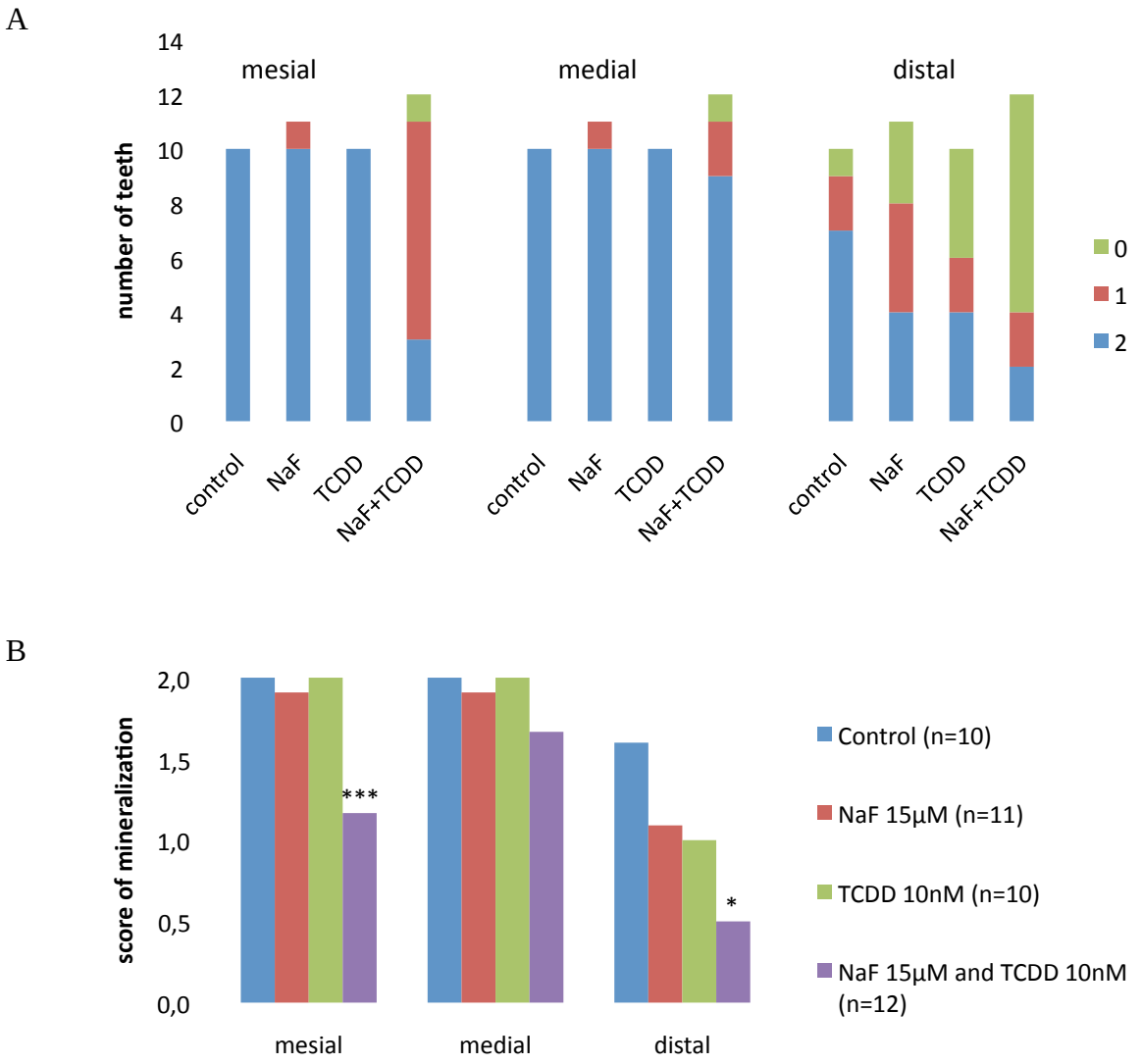

Figure 8. Mineralized dentin and enamel formation in the mesial, medial and distal cusps of first molars in control and exposure groups after 12 days of culture. Every cusp was given a score 2, 1 or 0 depending on the amount of hard tissue. Score 2 was given if there was a homogenous layer of mineralized dentin and enamel extending from the tip of the cusp to the cervical region. Score 1 was given if the amount of hard tissue was clearly reduced, and score 0 if the mineralization had not started at all. (A) The bars show the distribution of number of scores in each group. (B) The bars show mean values of the given scores of each group. Statistically significant difference from the control, NaF and TCDD groups is indicated by three asterisks; ***P < 0.01, Mann-Whitney test. Statistically significant difference from the control group is indicated by one asterisk; *P < 0.01, Mann-Whitney test.

Hematoxylin-eosin staining after 12 days of culture. - The enamel on the mesial slope of the first molar's mesial cusp was comparable to controls in teeth exposed to 15 µM NaF except for the intense purple staining in one of three teeth. All mesial ameloblasts were still columnar. The enamel was comparable to controls also in first molars exposed to 10 nM TCDD except for the strong staining in one of three teeth. Mesial ameloblasts in 2 of the 3 teeth were still columnar but lower than in the NaF-exposed teeth. HE-staining confirmed the stereomicroscopical findings: in teeth simultaneously exposed to 15 µM NaF and 10 nM TCDD, enamel thickness and extent were reduced. In addition, enamel stained darker than in

controls. Ameloblasts were columnar and partly detached from the enamel. The odontoblastic layer was somewhat disorganized.

4.2.3.2. Amelogenin expression in the first molar

Exposure to 10 μM NaF, 15 nM TCDD or their combination did not alter amelogenin expression in ameloblasts on the mesial slope of first molar mesial cusp after 7 days of culture. Immunostaining of odontoblasts in all three exposure groups was more intense than in controls. Amelogenin expression was also seen in dental papilla cells near the cusp tip in one of the two teeth exposed to NaF and TCDD simultaneously.

After 12 days of culture the intensity of amelogenin expression was comparable to controls in columnar, polarized ameloblasts in the cervical region on the mesial slope of the first molar mesial cusp where enamel matrix secretion was about to start or was in progress, and in the opposing polarized odontoblasts in first molars exposed to 10 μM NaF, 15 nM TCDD or to their combination. Staining was present in the still columnar (secretory) ameloblasts on the enamel surface in some teeth exposed to 10 μM NaF, to 10 nM TCDD, and to 15 μM NaF and 10 nM TCDD in combination. Amelogenin was also seen in all mesial odontoblasts extending from the cusp tip to the cervical region in part of teeth exposed to NaF and part of teeth exposed to NaF and TCDD simultaneously. Staining of odontoblasts in teeth exposed to TCDD corresponded to controls. Non-homogenous staining in dental papilla cells near the cusp tip was detected in part of teeth exposed to NaF and TCDD simultaneously.

4.2.3.3. Apoptosis in the first and second molars

Exposure to 10 μM and 20 μM NaF had no effect on apoptosis. Exposure to 15 nM TCDD slightly increased apoptosis in stellate reticulum and the oral epithelium but caused no apoptosis in the hard tissue forming cells, namely, ameloblasts and odontoblasts. The combined effect of NaF and TCDD did not differ from the weak effect of TCDD alone. For the statistical analysis of the amount of apoptosis in the hard tissue forming cells, apoptotic ameloblasts and odontoblasts in the mesial cusp of first molars were counted. There were no statistical differences between the groups ($P > 0.05$, Mann-Whitney test).

4.2.3.4. Cell proliferation in the first and second molars

12.5 μM NaF or 12.5 nM TCDD alone or in combination did not alter the rate of cell proliferation in the first molars. Cell proliferation, as estimated microscopically, in the first molar was so minimal that no statistical test was used.

For the statistical analysis of the amount of cell proliferation in the second molar tooth germ, all proliferating cells (epithelial and mesenchymal) in the cervical area were counted. There was no statistically significant difference ($P > 0.05$, Mann-Whitney test) between control and NaF or TCDD groups. Simultaneous exposure to NaF and TCDD reduced the frequency of labeled nuclei compared to controls. The difference in the amount of cell proliferation between control and NaF+TCDD group was statistically significant in the second molar ($P < 0.05$, Mann-Whitney test).

Table 2. Summary of the results

	DMBA		TBT		NaF+TCDD	
	1st	2nd	1st	2nd	1st	2nd
Reduced tooth size	↑ (ss)	↑	↑ (ss)			
Cuspal deformation	↑	↑	↑	x		
Thin predentin	↑	↑	↑	↑		
Impaired mineralization of dentin	↑	x	↑ (ss)	↑	x (ss)	x
Reduced amount of enamel	↑	x	↑ (ss)	↑	x (ss)	x
Dark enamel matrix / suggestive of impaired mineralization (intense HE-staining)					x	
Impaired elongation of ameloblasts	↑	↑	↑	↑		
Impaired elongation of odontoblasts			0	x		
Apoptosis			+	0	0	0
Cell proliferation			0	+	0	- (ss)
Ocn expression (QPCR)			+			
Ocn expression (*in situ* hybridization)			ob - epit. +			
Mmp-20 expression (QPCR)			-			
Mmp-20 expression (*in situ* hybridization)			ab - ob -			
Dspp expression (QPCR)			-			
Dspp expression (*in situ* hybridization)			ob -			
Amelogenin expression (immunohistochemistry)					ob + p +	

1st	first molar
2nd	second molar
↑	dose-dependent effect
x	effect observed, dose-dependency not studied
ss	statistically significant effect
0	no effect
+	increase
-	decrease
ob	odontoblasts
epit.	epithelial compartment of the tooth
ab	ameloblasts
p	dental papilla
gray cell	not in the scope of the study

5. DISCUSSION

5.1. General aspects (I, II, III, IV)

The results presented here provide *in vitro* confirmation of the study hypothesis that in addition to dioxins and dioxin-like compounds, other ubiquitous organic environmental toxicants, like non-halogenated polycyclic aromatic hydrocarbons (PAHs) and organic tin compounds, also have detrimental effects on tooth development, especially on the formation and mineralization of dentin and enamel. Furthermore, this work demonstrates that fluoride can potentiate the harmful effect of dioxins and dioxin-like compounds on dental hard tissue formation. Current knowledge about the effects of dioxins and dioxin-like compounds on human tooth development is mainly based on accidents, where large amounts of these harmful toxicants have been released into the environment. Yet, prevailing concentrations in the environment may be sufficiently high to impair tooth development. One of the suggested etiological factors of Molar-Incisor-Hypomineralization (MIH) is exposure to dioxins and dioxin-like compounds in early childhood (Alaluusua et al., 1996). PCDD/PCDF/PCB concentrations in mother's milk have been decreasing since the 1970s (Kiviranta, 2005; van Leeuwen and Malisch, 2002; Wilhelm et al., 2007), however the prevalence of MIH has not reduced in tandem as could have been expected (Weerheijm and Mejàre, 2003). The etiology of MIH is far from solved, however based on the results of the present thesis work, it is tangible to propose that other organic environmental toxicants, their combinations, and simultaneous exposure to fluoride may also impair dental hard tissue formation and mineralization in humans.

5.2. Methodological considerations (I, II, III, IV)

This thesis project was based on *in vitro* studies into the effects of various environmental toxicants and fluoride on dentin and enamel formation. The organ culture system used in the present work can be utilized to gain information about the effects and mechanisms of action of certain toxicants and fluoride on tooth development but exposure concentrations in organ cultures and *in vivo*, especially in humans, cannot be compared as such. As to the fluoride concentrations used in article IV, it can be estimated that the culture medium contained fluoride at the same order of magnitude as measured plasma levels in humans (Ekstrand, 1978). However, the actual TCDD concentration in the culture medium cannot be assessed because of the low solubility, poor penetration, and the tendency to adhere to surfaces of TCDD. The final exposure concentrations in organ cultures at the site of action are uncertain and probably much lower than concentrations used (Pohjanvirta and Tuomisto, 1994). The same problem may also concern DMBA and TBT.

There is a great difference in the duration of tooth development in human and mouse. Since the effects of DMBA, TBT, TCDD and fluoride on dental hard tissue formation depend on total exposure as calculated from the amount and duration of exposure, prolonged ingestion of lower amounts of these compounds by humans can have similar effects as a shorter exposure to slightly higher amounts by mice. Furthermore, humans are more susceptible to certain toxicants than rodents due to slower elimination (Van den Berg et al., 1994; Walton et al., 2001). Since the growth of the tooth explants and mineralization of dental matrices will set the limit to adequate nutrition of cells in organ culture, lower concentrations combined

with longer exposure times cannot be tested. Also, the homeostasis of a living organism is likely to modify the response of exposure to these toxicants as compared to explants deprived of their natural surroundings. Therefore, the clinical relevance of the present thesis project on the effects of DMBA, TBT and TCDD/fluoride on tooth development is largely speculative.

A feature common to dioxins, dioxin-like compounds, PAH compounds, and organotins is that they all are liposoluble and can be transferred to offspring through lactation. Because developing teeth are susceptible to each of these compounds, they could interfere with the formation of dental hard tissues not only separately but also in combination. Experimental dose response studies in different animal species and clinical observations in humans are needed to find out if there is any risk at prevailing levels. Regarding exposure to dioxins and dioxin-like compounds, the Finnish Food Safety Authority (Evira) has set recommendations on the consumption of fish products especially for fertile women and children.

5.3. Clinical relevance of exposure *in vitro*

5.3.1. Exposure route and timing (I, II, III, IV)

MIH is a condition in which there are demarcated enamel hypomineralization lesions in 1-4 permanent first molars and, frequently, also in incisors. Mineralization of the first permanent molars and incisors in humans begins around, or soon after, birth; the upper first incisors have completed their enamel formation by the end of the fifth year of life and first molars at about three years of age (Reid and Dean, 2006). Susceptibility of the enamel to developmental hypomineralization defects of environmental origin is highest at the transitional and early maturation stages of amelogenesis (Suga, 1989). Accordingly, human permanent incisors and first molars are at greatest risk for MIH up to the first years of life (Evans and Darvell, 1995; Moorrees et al., 1963). Therefore, lactational transfer of organic toxicants is a valid exposure route. It has been shown earlier that an infant can receive up to 25% of the mother's dioxin load *via* lactation (Vartiainen et al., 1997). Also PAH compounds and organotins may transfer to the infant by breast-feeding (Kimura et al., 2005; Zanieri et al., 2007). Children of heavy smokers living in urban areas may ingest PAH compounds *via* breast milk up to 1000 times more than the acceptable daily intake for drinking water established by the European commission (Zanieri et al., 2007). Furthermore, accumulation of these liposoluble agents in the child's fat may prolong the duration of their action. Regarding simultaneous exposure to fluoride, its content in mother's milk is rather low irrespective of concentration in drinking water (Chowdhury et al., 1990; Chuckpaiwong et al., 2000). However, infants acquire fluoride mainly from infant formulas diluted to water containing fluoride and by early use of fluoride toothpaste from the time of eruption of the first deciduous teeth (Osuji et al., 1988). Thus a breast-fed child getting fluorides from toothpaste already at an early age can be simultaneously exposed to organic toxicants and fluoride, with the exposure period overlapping with mineralization of the first permanent teeth.

In the present study design, exposure of the first molars was started at the late bell stage of morphogenesis, when differentiation of the dentin forming odontoblasts and enamel forming ameloblasts had started and deposition of dentin and enamel was about to begin. This stage corresponds well enough with the stage of human permanent first molars and incisors at birth. Therefore, from the clinical point of view, the organ culture system used in this thesis work was a valid model to experimentally investigate the interference in dental hard tissue formation caused by organic toxicants.

5.3.2. The effect on size and shape of tooth (I, II, IV)

In clinical studies, maternal smoking during pregnancy has been associated with size reduction of the child's deciduous as well as permanent teeth (Heikkinen et al., 1992; 1994a; 1994b; 1997). Delayed maturation of permanent teeth, that is, delayed dental age compared with chronological age has also been reported in children whose parents smoke (Kieser et al., 1996). Smoking increases the daily dietary intake of PAHs and further exposes the fetus to adverse effects of these compounds. After birth, infants can get even very high amounts of PAHs *via* breast-feeding (Zanieri et al., 2007). Results of our experimental study support the clinical observations and indicate that DMBA reduces tooth size. This is in line with the earlier findings that exposure of bank voles to PCDD/PCDF in contaminated sawmill area reduced the size of their third molars (Murtomaa et al., 2007), that molar and incisor tooth size was reduced in rats exposed *in vivo* to TCDD (Alaluusua et al., 1993; Kattainen et al., 2001; Lukinmaa et al., 2001; Miettinen et al., 2002), and that treatment of developing mouse mandibular molars with TCDD in organ culture resulted in a smaller tooth size (Partanen et al., 2004). We also demonstrated that first and second molars exposed to TBT were smaller than the corresponding control teeth.

This study has demonstrated an *in vitro* effect of one tobacco smoke component, DMBA, on tooth development. Tobacco smoke also contains several other toxic compounds like nicotine, carbon monoxide, nitrogen oxides, and hydrogen cyanide (Hoffmann et al., 2001), which may have adverse effects of their own on tooth development. Nicotine has been shown to retard tooth development and to reduce the size of the first molar in rats and mice *in vivo* (Chowdhury and Bromage, 2000; Saad et al., 1991). *In vitro*, nicotine exposure causes necrosis in the dental papilla, and when this involves the odontoblast layer, the amount of predentin is reduced (Khan et al., 1981). Nicotine also impairs the secretion of enamel matrix (Khan et al., 1981). Thus, nicotine and PAH compounds seem to have similar effects that may additively impair tooth development.

Exposure to DMBA also altered cuspal morphology, and the effect was more severe in the second than in the first molar. Narrow and curved cusps are also observed in mouse E18 first molars cultured for 11 days with TCDD (Partanen et al., 1998). After TBT exposure reduced or lacking predentin in the developmentally less advanced medial and distal cusps of the first molars was accompanied by morphological alteration in the stellate reticulum of the enamel organ and deterioration of the cusps. The stellate reticulum cells lost their normal star-like shape and the loose-textured tissue became a dense mass of enlarged eosinophilic cells with round, central nuclei, possibly indicating an incipient cell death. In the second molars thinner predentin and cusps were observed but the changes in the stellate reticulum were less obvious than in the first molars.

In the study of the effects of NaF and TCDD on tooth development (IV) their concentrations were so low that neither agent had effect on tooth size when administered separately. However, the second molars exposed to NaF and TCDD in combination appeared to be smaller than controls. This supports the hypothesis that the agents may potentiate each other's effect.

Rather than a specific effect, size reduction and altered cuspal morphology may indicate a general toxic effect of the studied toxicants on organ development.

5.4. The effects on dentin and enamel formation and mineralization (I, II, IV)

The main focus of this thesis project was on the toxic effects of DMBA, TBT, NaF and TCDD on dentin and enamel formation. Exposure to PCDDs and PCDFs has been connected with enamel defects in humans (Alaluusua et al., 1996; 2004). It is also well known that excess fluoride intake during tooth development causes fluorosis, a specific type of enamel hypomineralization. On the other hand, the frequency of enamel defects in the permanent first molars was not found to be increased in children whose mothers had smoked during the last 12 months before delivery (Alaluusua et al., 1996), even if a number of different PAHs, such as benzo[a]pyrene (BP) and benz[a]anthracene, are among the main toxic components of tobacco smoke (Hoffmann et al., 2001).

The results of the present thesis project showed that DMBA and TBT, a trisubstituted organotin, impair mineralization of dentin and formation of enamel in cultured mouse E18 mandibular first molars. Depending on the concentration, the effect ranged from barely detectable to a complete absence of mineralized dentin and enamel. When predentin had not been mineralized and enamel had not been formed in the mesial cusps of the first molars, ameloblasts were not elongated but had remained cuboidal, which likely represents a morphological sign of their impaired function. Where predentin formation had not started in the second molars, ameloblasts and odontoblasts were nonpolarized, which in turn, was suggestive of their impaired differentiation.

DMBA is a representative of a class of toxic non-halogenated PAHs. No *in vivo* or *in vitro* studies, on the effects of PAH compounds, or organic tin compounds, on tooth development appear to have been conducted before the present thesis project. Previous studies show, however, that TBT interferes with biomineralization in different species (Adeeko et al., 2003; Chagot et al., 1990; Suzuki et al., 2006; Tsukamoto et al., 2004). Response of the process ranges from inhibited differentiation and activity of osteoblasts to impaired deposition of calcium, and finally to delayed ossification of bones (Adeeko et al., 2003; Suzuki et al., 2006; Tsukamoto et al., 2004). Calcium deposition is also essential for mineralization of predentin to dentin (Linde and Lundgren, 1995; Woltgens et al., 1987). Accordingly, we found that TBT interferes with predentin mineralization and enamel formation.

Notwithstanding that fluoride and dioxin are completely different compounds chemically, they both interfere with dental hard tissue formation. In the present study we showed that simultaneous exposure of cultured mouse embryonic molars to NaF and TCDD at concentrations, 15 μM and 10 nM, respectively, which alone had no or a barely detectable effect on tooth development, significantly impaired dentin mineralization and enamel matrix deposition. In histological tissue sections stained with hematoxylin and eosin (HE), the enamel consistently stained darker than in controls suggesting retention of enamel matrix proteins and accordingly hypomineralization. The presence of enamel matrix could be due to delayed development or the adverse effect of NaF and TCDD on enamel mineralization.

Earlier studies have demonstrated that exposure of cultured mouse tooth germs to TCDD during the formation of dental hard tissues impairs dentin mineralization and arrests enamel formation (Partanen et al., 1998). TCDD exposure of rat pups *via* lactation arrested the deposition of dentin and enamel matrices (Lukinmaa et al., 2001), impaired dentin mineralization and delayed degradation of enamel matrix during enamel maturation in developing molar teeth (Gao et al., 2004). In a previous *in vitro* study, in another mouse

strain, the adverse effects of TCDD occasionally became evident at a concentration of 0.5 μM and were consistent at 1.0 μM (Partanen et al., 1998). In the present study, effects of TCDD on dental hard tissue formation, albeit barely detectable, were occasionally seen at a concentration as low as 10 nM. The sensitivity difference could be explained by the mouse strain difference. On the other hand, we did not find differences in the response to TCDD exposure of the continuously erupting incisors between the highly resistant H/W and the sensitive LE rat strains (Kiukkonen et al., 2002).

Fluorosis is thought to involve defective degradation and removal of amelogenins from the enamel matrix, which is needed to create space for the mineral phase (Den Besten, 1986). As such, after exposure to 15 μM NaF, the enamel stained dark purple in HE staining, albeit inconsistently. Although 15 μM NaF did not affect the amount of enamel matrix, in accordance with earlier findings by Bronckers et al. (1984), we observed that at higher concentrations fluoride reduced the thickness of the enamel matrix. As shown previously by Bronckers et al. (1984), we further observed that 15 μM NaF accentuated the globular shape of the mineralization front of dentin, suggesting a sparse distribution of mineralization nodules. Consistent with this finding, NaF at higher concentrations delayed dentin mineralization.

5.5. Mechanistic aspects

5.5.1. The role of epithelial-mesenchymal interactions (I, II, IV)

A requirement for the differentiation of the ectomesenchyme-derived odontoblasts is a signal from the epithelial preameloblasts. For the differentiation of ameloblasts, a signal from odontoblasts is needed and ameloblasts do not become secretory until odontoblasts have laid down a thin layer of predentin and it has started to mineralize (He et al., 2010; Tompkins, 2006). Launching of the secretory activity of ameloblasts by the start of dentin matrix mineralization represents the final step in the series of epithelial-mesenchymal interactions that instruct tooth crown development (Thesleff, 2003; Tompkins, 2006). Therefore, the failure of ameloblasts to become secretory or the impaired secretory capacity of ameloblasts could be secondary to the adverse effect of the studied toxicants on predentin mineralization.

While earlier studies suggest that fluoride causes enamel hypomineralization by affecting enamel directly, the impaired or delayed enamel matrix secretion and mineralization could as well be secondary to the inhibitory effect of NaF on the mineralization of predentin to dentin. Given that TCDD has a similar effect, the combined impact of NaF and TCDD on amelogenesis could also be a consequence of retarded dentin mineralization.

5.5.2. The effects at the cellular level: apoptosis and cell proliferation (II, IV)

Apoptosis, or programmed cell death, plays a central role in the normal development of various organ systems by controlling the number of cells (Vaux and Korsmeyer, 1999). Developmental disorders and pathological processes can also involve apoptosis. Various cell types of the dental epithelium also undergo apoptosis in the course of normal tooth development: Apoptotic cells have been localized in the dental lamina, the epithelial cord connecting the early tooth germ to the oral epithelium (Vaahtokari et al., 1996). The enamel knots, which are transient signaling centers that guide cuspal morphogenesis, also disappear

apoptotically (Vaahtokari et al., 1996). Up to 50% of ameloblasts may undergo apoptosis during enamel maturation (Smith and Warshawsky, 1977) and the number of cells of the stratum intermedium and stellate reticulum will significantly reduce by apoptosis during advancing enamel formation (Vaahtokari et al., 1996), to disappear upon tooth eruption.

Those dental epithelial cells that are predestined to undergo apoptosis may respond to a toxic insult by entering apoptotic cell death precociously. The arrest of early mouse tooth development and cuspal deformation after TCDD exposure is known to involve precocious and increased apoptosis in the dental lamina and enamel knots, respectively (Partanen et al., 2004). The results of the present thesis work showed that also after TBT exposure, apoptosis was enhanced in the dental epithelium programmed to die apoptotically, although the effect was studied at a later stage of the first and second molar development. Even though the reduced size of the first molar due to TBT-exposure can partly be explained by lacking or thin enamel, apoptosis in the enamel organ may have contributed to deterioration of the cusp shape and to smaller tooth size. Reduction of tooth size after DMBA treatment could also have involved acceleration and/or increase of apoptotic death of dental epithelial cells, since PAH compounds are known to induce the expression of an apoptosis-regulating gene, the *Bax* gene (Matikainen et al., 2001; 2002). Fluoride has also been shown to increase apoptosis in the ameloblast-like cells in zebrafish (Bartlett et al., 2005) but not in cultured odontoblast-like cells (Wurtz et al., 2008).

The mechanism behind the detrimental effects of exposure to higher concentrations of the toxicants studied or exposure at early stages of tooth development is supposed to involve increased apoptosis. However, as shown in article IV, where developing teeth were exposed to very low concentrations of fluoride and TCDD, neither 20 μM NaF nor 15 nM TCDD or their combination enhanced apoptosis of secretory ameloblasts or odontoblasts. Therefore, mineralization defects could not be attributed to increased apoptosis of dental hard tissue - forming cells. Thus, the present results do not suggest a role for apoptosis in impairing dental hard tissue formation during mineralization or maturation stages after exposure to organic environmental toxicants and fluoride at low concentrations.

Once the dental cells have differentiated, they no longer proliferate. In this thesis project the lower first molars were at the late bell stage of morphogenesis when the experiments were started and accordingly after 5 days of culture, cell proliferation was ongoing only in the cervical area. Therefore reduced tooth size or impaired hard tissue formation in the first molar could not have resulted from altered cell proliferation. However, corresponding to the earlier stage of tooth development, proliferation was more abundant in the second than in the first molars. After TBT exposure proliferating cells were more frequent in both the epithelial and mesenchymal tissues of the exposed second molar than in controls. Abundant cell proliferation in the second molars is consistent with the morphological and functional signs of retarded differentiation. On the other hand, simultaneous exposure to NaF and TCDD significantly reduced proliferation in the second molar. The second molars exposed to NaF and TCDD in combination seemed to be smaller than controls. Therefore, delayed or impaired tooth development may also be explained by reduced cell proliferation.

5.5.3. The effect at the protein level: amelogenin expression (IV)

We studied if altered amelogenin expression after NaF and TCDD exposure would explain impaired enamel formation. The results showed that NaF and TCDD not only together but also alone interfered with the pattern of amelogenin expression in ameloblasts and

odontoblasts. Morphology of ameloblasts and the expression of amelogenin indicate that, in contrast to the controls, ameloblasts were still secretory and had not entered the maturation stage, suggesting delayed secretion and maturation processes of enamel.

Both ameloblasts and odontoblasts express amelogenin (Papagerakis et al., 2003; Tompkins et al., 2005). Amelogenin expression by odontoblasts was increased in response to NaF and TCDD both alone and together. Tompkins et al. (2005) hypothesized that an amelogenin isoform [A-4] produced by secretory odontoblasts normally delays conversion of preameloblasts to secretory ameloblasts until a sufficiently thick layer of dentin has been produced. Therefore, a reason for the impaired enamel formation after simultaneous NaF and TCDD exposure could be delayed differentiation of ameloblasts due to increased amelogenin expression by odontoblasts. In a previous study, Bronckers et al. (1998) concluded that the pathological absence of mineralized dentin and enamel allows amelogenin from secretory ameloblasts to pass the predentin and odontoblastic layers and to accumulate in the pulp. Accordingly, we observed amelogenin reactivity in the dental papilla cells after simultaneous exposure to NaF and TCDD. However, since mineralized dentin and enamel were present, amelogenin in the dental papilla cells was probably not a product of ameloblasts but likely to have been secreted by the mesenchymal dental papilla cells themselves.

Since the number of specimens was rather small, any definite conclusions cannot be drawn based on these results.

5.5.4. The effects on gene expression (III)

In this thesis project we demonstrated by QPCR that exposure to TBT increases *osteocalcin* (*Ocn*) expression in cultured mouse embryonic first molars during the secretory and mineralization stages of development. The earlier the exposure was started the clearer the QPCR-results: when the explants were cultured the first two days without TBT and then exposed for 3 or 5 days, the results were non-uniform. However, *in situ* hybridization showed that while increasing expression in the epithelial compartment of the tooth TBT exposure decreases *Ocn* gene expression in odontoblasts. We also found that exposure of the explants to TBT reduces *Mmp-20* gene expression both in ameloblasts and odontoblasts. The effect of TBT on *dentin sialophosphoprotein* (*Dspp*) expression varied but both QPCR experiments and *in situ* hybridization showed a decreasing trend.

Thus far, the exact role of Ocn has not been defined. Tsukamoto et al. (2004) observed that TBT decreases *Ocn* expression in osteoblasts. They concluded that TBT impairs differentiation of osteoblasts and that the reduced *Ocn* expression is a marker of this effect. Corresponding to the effect on osteoblasts we found that *Ocn* expression is also decreased in odontoblasts, which suggests that TBT delays differentiation of odontoblasts as well. By *in situ* hybridization we observed that whereas *Ocn* expression in odontoblasts was decreased, it was non-uniformly increased in ameloblasts, stratum intermedium and stellate reticulum cells. The increase in *Ocn* expression observed by QPCR can be explained by the proportional predominance of the epithelial component of the tooth over the mesenchymal odontoblasts. Ocn inhibits HA crystal nucleation and growth *in vitro* (Hunter et al., 1996; Romberg et al., 1986). Furthermore, exogenous Ocn is capable of impairing the formation of mineralized dentin and enamel (Bronckers et al., 1998), and Ocn is also expressed by dental epithelial cells in areas where enamel is not deposited on the mineralized dentin (Bosshardt and Nanci, 1997). Therefore, the increased *Ocn* expression by dental epithelial cells after TBT exposure, may have contributed to the inhibition of normal dentin mineralization and of

enamel formation as shown in our study on morphological effects of TBT on mouse embryonic tooth explants.

DSPP has an important role in the formation of mineralized dentin. Suzuki et al. (2009) suggested that one of the three DSPP domains, dentin sialoprotein (DSP), predominantly regulates initiation of dentin mineralization, whereas another domain, dentin phosphoprotein (DPP), is primarily involved in the maturation of mineralized dentin. Teeth of *Dspp* knockout mice display a widened predentin zone and defective dentin mineralization (Sreenath et al., 2003). In a previous study, as shown by *in situ* hybridization, TCDD decreased *Dspp* gene expression in cultured mouse embryonic teeth (Kiukkonen et al., 2006). Accordingly, the effect of TBT on *Dspp* expression showed a decreasing trend in QPCR and reduced expression was observed by *in situ* hybridization. The results suggested that impaired dentin mineralization due to TBT exposure could have involved altered *Dspp* expression.

MMP-20 (enamelysin) is a proteinase, which degrades organic enamel matrix during the mineralization of enamel (Caterina et al., 2002; Hu et al., 2002). Further, MMP-20 cleaves DSP from dentin glycoprotein (DGP) and also generates a series of DSP-positive cleavage products (Yamakoshi et al. 2006). Knocking out *Mmp-20* in mice resulted in a thinner than normal enamel layer, which lacked normal rod (prism) structure, tended to delaminate from the underlying dentin and had decreased mineral content (Bartlett et. al, 2004; Caterina et al., 2002). Inhibition of MMP activity impaired enamel formation and mineralization in cultured mouse embryonic molars (Bourd-Boittin et al., 2005). These results greatly resemble our morphological findings on effects of organic environmental toxicants on dental hard tissue formation. In our earlier study TCDD was not found to alter *Mmp-20* expression in ameloblasts of cultured mouse embryonic teeth, but in odontoblasts the expression was slightly decreased (Kiukkonen et al., 2006). In the present project there was a clear decrease in *Mmp-20* expression after TBT-exposure in ameloblasts and odontoblasts. Decreased *Mmp-20* expression in odontoblasts may have hindered processing of DSPP and thereby impaired dentin mineralization. Furthermore, decreased *Mmp-20* expression in ameloblasts is likely to have directly impaired enamel matrix degradation required for mineralization. Total proteinase activity has also been shown to decrease in the enamel matrix of fluoride-exposed rats during enamel maturation, and specifically the activity of MMP-20 has been observed to reduce in the presence of fluoride *in vitro* (DenBesten et al., 2002). Thus, a mechanism common to fluoride and TCDD in the inhibition of mineralization could be a decrease in *Mmp-20* gene expression. This hypothesis is further supported by the finding that tooth germs treated with an MMP inhibitor marimastat showed increased immunostaining for amelogenin and the distribution pattern of the protein was altered and diffuse as in our NaF/TCDD-experiment (Bourd-Boittin et al., 2005).

6. CONCLUSIONS

The results of this thesis project have shown that DMBA, TBT, TCDD and NaF interfere with hard tissue formation of embryonic mouse tooth *in vitro*. The effects are concentration and developmental stage dependent with the susceptibility decreasing with advancing tooth development.

This study confirms the toxic influence of non-halogenated PAHs on tooth development and supports the earlier results of tooth size reduction caused by maternal smoking during pregnancy. These results are also in line with previous findings concerning the effects of TBT on bone. This work provides experimental evidence that the developmental toxicity of fluoride and dioxin is potentiative. Children may be exposed to subclinical levels of fluoride and dioxins during early childhood. Since the exposure time coincides with mineralization of the first permanent teeth, this finding may have clinical significance.

Decreased *Ocn*, *Mmp-20* and *Dspp* expressions in odontoblasts after TBT-exposure may indicate delayed cell differentiation or alternatively, TBT may specifically decrease expression of genes involved in mineralization of dentin. Decrease in *Mmp-20* expression by TBT in ameloblasts is likely to directly impair enamel mineralization. The coincident reduction of *Mmp-20* and *Dspp* expressions in odontoblasts may have potentiated the delay of dentin mineralization. Since the morphological findings after DMBA and TCDD exposure of tooth explants resemble those seen after TBT exposure, these results and their interpretations may also apply to DMBA and TCDD.

The results support the hypothesis that DMBA and TBT as well as NaF and TCDD together primarily affect odontoblasts and/or dentin mineralization. Since amelogenesis does not start until mineralization of predentin to dentin begins, impaired enamel matrix secretion by ameloblasts could be secondary to the inhibitory effect of the toxicants on dentin mineralization.

The major dental hard tissues, the dentin and the enamel, are not replaced once they have been formed. Therefore, tooth may act as an indicator of exposure to environmental toxins. Disturbed dental hard tissue formation raises the question of more extensive developmental toxicity of these compounds.

Tooth development has not been routinely studied in regulatory safety evaluation studies of pharmaceuticals, pesticides and other potential toxins. The present thesis work showed that structurally and mechanistically different chemicals impair tooth development at relatively low concentrations. Therefore, suitability of certain endpoints of tooth development, such as tooth size or dental hard tissue mineralization could be evaluated as novel and potentially sensitive targets of dental toxicity and possibly also as general indicators of developmental toxicity.

REFERENCES

Abe, K., Ooshima, T., Lily, T. S., Yasufuku, Y., Sobue, S. Structural deformities of deciduous teeth in patients with hypophosphatemic vitamin D-resistant rickets. *Oral Surg. Oral Med. Oral Pathol.* 1988; 65: 191-198.

Aberg, T., Wozney, J., Thesleff, I. Expression patterns of bone morphogenetic proteins (bmps) in the developing mouse tooth suggest roles in morphogenesis and cell differentiation. *Dev. Dyn.* 1997; 210: 383-396.

Adeeko, A., Li, D., Forsyth, D. S., Casey, V., Cooke, G. M., Barthelemy, J., Cyr, D. G., Trasler, J. M., Robaire, B., Hales, B. F. Effects of in utero tributyltin chloride exposure in the rat on pregnancy outcome. *Toxicol. Sci.* 2003; 74: 407-415.

Aine, L. Dental enamel defects and dental maturity in children and adolescents with coeliac disease. *Proc. Finn. Dent. Soc.* 1986; 82 Suppl 3: 1-71.

Alaluusua, S., Calderara, P., Gerthoux, P. M., Lukinmaa, P. L., Kovero, O., Needham, L., Patterson, D. G.,Jr, Tuomisto, J., Mocarelli, P. Developmental dental aberrations after the dioxin accident in seveso. *Environ. Health Perspect.* 2004; 112: 1313-1318.

Alaluusua, S., Lukinmaa, P. L., Pohjanvirta, R., Unkila, M., Tuomisto, J. Exposure to 2,3,7,8-tetrachlorodibenzo-para-dioxin leads to defective dentin formation and pulpal perforation in rat incisor tooth. *Toxicology* 1993; 81: 1-13.

Alaluusua, S., Lukinmaa, P. L., Vartiainen, T., Partanen, M., Torppa, J., Tuomisto, J. Polychlorinated dibenzo-*p*-dioxins and dibenzofurans via mother's milk may cause developmental defects in the child's teeth. *Environ Toxicol Pharmacol* 1996; 1: 193-197.

Alzieu, C. Environmental impact of TBT: The french experience. *Sci. Total Environ.* 2000; 258: 99-102.

Antizar-Ladislao, B. Environmental levels, toxicity and human exposure to tributyltin (TBT)-contaminated marine environment. a review. *Environ. Int.* 2008; 34: 292-308.

Aoba, T., Shimoda, S., Akita, H., Holmberg, C., Taubman, M. A. Anti-peptide antibodies reactive with epitopic domains of porcine amelogenins at the C-terminus. *Arch. Oral Biol.* 1992; 37: 249-255.

Argiro, L., Desbarats, M., Glorieux, F. H., Ecarot, B. Mepe, the gene encoding a tumor-secreted protein in oncogenic hypophosphatemic osteomalacia, is expressed in bone. *Genomics* 2001; 74: 342-351.

Arnould, J. P., Verhoest, P., Bach, V., Libert, J. P., Belegaud, J. Detection of benzo[a]pyrene-DNA adducts in human placenta and umbilical cord blood. *Hum. Exp. Toxicol.* 1997; 16: 716-721.

Arsenault, A. L. and Robinson, B. W. The dentino-enamel junction: A structural and microanalytical study of early mineralization. *Calcif. Tissue Int.* 1989; 45: 111-121.

Baht, G. S., Hunter, G. K., Goldberg, H. A. Bone sialoprotein-collagen interaction promotes hydroxyapatite nucleation. *Matrix Biol.* 2008; 27: 600-608.

Bailleul-Forestier, I., Berdal, A., Vinckier, F., de Ravel, T., Fryns, J. P., Verloes, A. The genetic basis of inherited anomalies of the teeth. Part 2: syndromes with significant dental involvement. *Eur. J. Med. Genet.* 2008; 51: 383-408.

Barron, M. J., McDonnell, S. T., Mackie, I., Dixon, M. J. Hereditary dentine disorders: Dentinogenesis imperfecta and dentine dysplasia. *Orphanet J. Rare Dis.* 2008; 3: 31.

Bartlett, J. D., Beniash, E., Lee, D. H., Smith, C. E. Decreased mineral content in MMP-20 null mouse enamel is prominent during the maturation stage. *J. Dent. Res.* 2004; 83: 909-913.

Bartlett, J. D., Dwyer, S. E., Beniash, E., Skobe, Z., Payne-Ferreira, T. L. Fluorosis: A new model and new insights. *J. Dent. Res.* 2005; 84: 832-836.

Beertsen, W., VandenBos, T., Everts, V. Root development in mice lacking functional tissue non-specific alkaline phosphatase gene: Inhibition of acellular cementum formation. *J. Dent. Res.* 1999; 78: 1221-1229.

Begue-Kirn, C., Smith, A. J., Ruch, J. V., Wozney, J. M., Purchio, A., Hartmann, D., Lesot, H. Effects of dentin proteins, transforming growth factor beta 1 (TGF beta 1) and bone morphogenetic protein 2 (BMP2) on the differentiation of odontoblast in vitro. *Int. J. Dev. Biol.* 1992; 36: 491-503.

Bei, M., Kratochwil, K., Maas, R. L. BMP4 rescues a non-cell-autonomous function of Msx1 in tooth development. *Development* 2000; 127: 4711-4718.

Bidder, M., Latifi, T., Towler, D. A. Reciprocal temporospatial patterns of Msx2 and osteocalcin gene expression during murine odontogenesis. *J. Bone Miner. Res.* 1998; 13: 609-619.

Birnbaum, L. S. and Tuomisto, J. Non-carcinogenic effects of TCDD in animals. *Food Addit. Contam.* 2000; 17: 275-288.

Bleicher, F., Couble, M. L., Farges, J. C., Couble, P., Magloire, H. Sequential expression of matrix protein genes in developing rat teeth. *Matrix Biol.* 1999; 18: 133-143.

Boskey, A., Spevak, L., Tan, M., Doty, S. B., Butler, W. T. Dentin sialoprotein (DSP) has limited effects on in vitro apatite formation and growth. *Calcif. Tissue Int.* 2000; 67: 472-478.

Boskey, A. L., Gadaleta, S., Gundberg, C., Doty, S. B., Ducy, P., Karsenty, G. Fourier transform infrared microspectroscopic analysis of bones of osteocalcin-deficient mice provides insight into the function of osteocalcin. *Bone* 1998; 23: 187-196.

Boskey, A. L., Spevak, L., Doty, S. B., Rosenberg, L. Effects of bone CS-proteoglycans, DS-decorin, and DS-biglycan on hydroxyapatite formation in a gelatin gel. *Calcif. Tissue Int.* 1997; 61: 298-305.

Boskey, A. L., Spevak, L., Paschalis, E., Doty, S. B., McKee, M. D. Osteopontin deficiency increases mineral content and mineral crystallinity in mouse bone. *Calcif. Tissue Int.* 2002; 71: 145-154.

Bosshardt, D. D. Are cementoblasts a subpopulation of osteoblasts or a unique phenotype? *J. Dent. Res.* 2005; 84: 390-406.

Bosshardt, D. D. and Nanci, A. Immunodetection of enamel- and cementum-related (bone) proteins at the enamel-free area and cervical portion of the tooth in rat molars. *J. Bone Miner. Res.* 1997; 12: 367-379.

Bourd-Boittin, K., Fridman, R., Fanchon, S., Septier, D., Goldberg, M., Menashi, S. Matrix metalloproteinase inhibition impairs the processing, formation and mineralization of dental tissues during mouse molar development. *Exp. Cell Res.* 2005; 304: 493-505.

Bronckers, A. L., Gay, S., Finkelman, R. D., Butler, W. T. Developmental appearance of gla proteins (osteocalcin) and alkaline phosphatase in tooth germs and bones of the rat. *Bone Miner.* 1987; 2: 361-373.

Bronckers, A. L., Jansen, L. L., Woltgens, J. H. Long-term (8 days) effects of exposure to low concentrations of fluoride on enamel formation in hamster tooth-germs in organ culture in vitro. *Arch. Oral Biol.* 1984; 29: 811-819.

Bronckers, A. L., Lyaruu, D. M., DenBesten, P. K. The impact of fluoride on ameloblasts and the mechanisms of enamel fluorosis. *J. Dent. Res.* 2009; 88: 877-893.

Bronckers, A. L., Price, P. A., Schrijvers, A., Bervoets, T. J., Karsenty, G. Studies of osteocalcin function in dentin formation in rodent teeth. *Eur. J. Oral Sci.* 1998; 106: 795-807.

Butler, W. T., Brunn, J. C., Qin, C. Dentin extracellular matrix (ECM) proteins: Comparison to bone ECM and contribution to dynamics of dentinogenesis. *Connect. Tissue Res.* 2003; 44 Suppl 1: 171-178.

Caramaschi, F., del Corno, G., Favaretti, C., Giambelluca, S. E., Montesarchio, E., Fara, G. M. Chloracne following environmental contamination by TCDD in seveso, italy. *Int. J. Epidemiol.* 1981; 10: 135-143.

Caterina, J. J., Skobe, Z., Shi, J., Ding, Y., Simmer, J. P., Birkedal-Hansen, H., Bartlett, J. D. Enamelysin (matrix metalloproteinase 20)-deficient mice display an amelogenesis imperfecta phenotype. *J. Biol. Chem.* 2002; 277: 49598-49604.

Chagot, D., Alzieu, C., Sanjuan, J., Grizel, H. Sublethal and histopathological effects of trace levels of tributyltin fluoride on adult oysters *crassostrea gigas*. *Aquat. Living Resour.* 1990; 3: 121-130.

Chang, S. R., Chiego, D.,Jr, Clarkson, B. H. Characterization and identification of a human dentin phosphophoryn. *Calcif. Tissue Int.* 1996; 59: 149-153.

Chapple, I. L. Hypophosphatasia: Dental aspects and mode of inheritance. *J. Clin. Periodontol.* 1993; 20: 615-622.

Chen, B., Goodman, E., Lu, Z., Bandyopadhyay, A., Magraw, C., He, T., Raghavan, S. Function of beta1 integrin in oral epithelia and tooth bud morphogenesis. *J. Dent. Res.* 2009; 88: 539-544.

Chen, S., Chen, L., Jahangiri, A., Chen, B., Wu, Y., Chuang, H. H., Qin, C., MacDougall, M. Expression and processing of small integrin-binding ligand N-linked glycoproteins in mouse odontoblastic cells. *Arch. Oral Biol.* 2008; 53: 879-889.

Chen, S., Rani, S., Wu, Y., Unterbrink, A., Gu, T. T., Gluhak-Heinrich, J., Chuang, H. H., Macdougall, M. Differential regulation of dentin sialophosphoprotein expression by Runx2 during odontoblast cytodifferentiation. *J. Biol. Chem.* 2005; 280: 29717-29727.

Chowdhury, I. G. and Bromage, T. G. Effects of fetal exposure to nicotine on dental development of the laboratory rat. *Anat. Rec.* 2000; 258: 397-405.

Chowdhury, N. G., Brown, R. H., Shepherd, M. G. Fluoride intake of infants in new zealand. *J. Dent. Res.* 1990; 69: 1828-1833.

Chuckpaiwong, S., Nakornchai, S., Surarit, R., Soo-ampon, S. Fluoride analysis of human milk in remote areas of thailand. *Southeast Asian J. Trop. Med. Public Health* 2000; 31: 583-586.

Cooke, G. M. Effect of organotins on human aromatase activity in vitro. *Toxicol. Lett.* 2002; 126: 121-130.

David, V., Martin, A., Hedge, A. M., Drezner, M. K., Rowe, P. S. ASARM peptides: PHEX-dependent and -independent regulation of serum phosphate. *Am. J. Physiol. Renal Physiol.* 2011; 300: F783-791.

Davideau, J. L., Sahlberg, C., Blin, C., Papagerakis, P., Thesleff, I., Berdal, A. Differential expression of the full-length and secreted truncated forms of EGF receptor during formation of dental tissues. *Int. J. Dev. Biol.* 1995; 39: 605-615.

Deakins, M. and Volker, J. F. Amount of organic matter in enamel from several types of human teeth. *J. Dent. Res.* 1941; 20: 117-121.

Den Besten, P. K. Effects of fluoride on protein secretion and removal during enamel development in the rat. *J. Dent. Res.* 1986; 65: 1272-1277.

DenBesten, P. K., Yan, Y., Featherstone, J. D., Hilton, J. F., Smith, C. E., Li, W. Effects of fluoride on rat dental enamel matrix proteinases. *Arch. Oral Biol.* 2002; 47: 763-770.

Diekwisch, T. G. The developmental biology of cementum. *Int. J. Dev. Biol.* 2001; 45: 695-706.

D'Souza, R. N., Cavender, A., Sunavala, G., Alvarez, J., Ohshima, T., Kulkarni, A. B., MacDougall, M. Gene expression patterns of murine dentin matrix protein 1 (Dmp1) and dentin sialophosphoprotein (DSPP) suggest distinct developmental functions in vivo. *J. Bone Miner. Res.* 1997; 12: 2040-2049.

Ducy, P., Desbois, C., Boyce, B., Pinero, G., Story, B., Dunstan, C., Smith, E., Bonadio, J., Goldstein, S., Gundberg, C., Bradley, A., Karsenty, G. Increased bone formation in osteocalcin-deficient mice. *Nature* 1996; 382: 448-452.

Econs, M. J. and Francis, F. Positional cloning of the PEX gene: New insights into the pathophysiology of X-linked hypophosphatemic rickets. *Am. J. Physiol.* 1997; 273: F489-98.

Egeland, G. M., Sweeney, M. H., Fingerhut, M. A., Wille, K. K., Schnorr, T. M., Halperin, W. E. Total serum testosterone and gonadotropins in workers exposed to dioxin. *Am. J. Epidemiol.* 1994; 139: 272-281.

Ekstrand, J. Relationship between fluoride in the drinking water and the plasma fluoride concentration in man. *Caries Res.* 1978; 12: 123-127.

Ema, M., Harazono, A., Miyawaki, E., Ogawa, Y. Effect of the day of administration on the developmental toxicity of tributyltin chloride in rats. *Arch. Environ. Contam. Toxicol.* 1997; 33: 90-96.

Embery, G., Hall, R., Waddington, R., Septier, D., Goldberg, M. Proteoglycans in dentinogenesis. *Crit. Rev. Oral Biol. Med.* 2001; 12: 331-349.

Embery, G., Rees, S., Hall, R., Rose, K., Waddington, R., Shellis, P. Calcium- and hydroxyapatite-binding properties of glucuronic acid-rich and iduronic acid-rich glycosaminoglycans and proteoglycans. *Eur. J. Oral Sci.* 1998; 106 Suppl 1: 267-273.

European Food Safety Authority. Opinion of the Scientific Panel on Contaminants in the Food Chain on a request from the Commission to assess the health risks to consumers associated with exposure to organotins in foodstuffs. *EFSA J.* 2004; 102: 1–119.

Evans, R. W. and Darvell, B. W. Refining the estimate of the critical period for susceptibility to enamel fluorosis in human maxillary central incisors. *J. Public Health Dent.* 1995; 55: 238-249.

Everett, E. T., McHenry, M. A., Reynolds, N., Eggertsson, H., Sullivan, J., Kantmann, C., Martinez-Mier, E. A., Warrick, J. M., Stookey, G. K. Dental fluorosis: Variability among different inbred mouse strains. *J. Dent. Res.* 2002; 81: 794-798.

Fan, D., Du, C., Sun, Z., Lakshminarayanan, R., Moradian-Oldak, J. In vitro study on the interaction between the 32 kDa enamelin and amelogenin. *J. Struct. Biol.* 2009; 166: 88-94.

Fejerskov, O., Johnson, N. W., Silverstone, L. M. The ultrastructure of fluorosed human dental enamel. *Scand. J. Dent. Res.* 1974; 82: 357-372.

Fejerskov, O., Larsen, M. J., Richards, A., Baelum, V. Dental tissue effects of fluoride. *Adv. Dent. Res.* 1994; 8: 15-31.

Feng, J. Q., Ward, L. M., Liu, S., Lu, Y., Xie, Y., Yuan, B., Yu, X., Rauch, F., Davis, S. I., Zhang, S., Rios, H., Drezner, M. K., Quarles, L. D., Bonewald, L. F., White, K. E. Loss of DMP1 causes rickets and osteomalacia and identifies a role for osteocytes in mineral metabolism. *Nat. Genet.* 2006; 38: 1310-1315.

Fernandez-Salguero, P. M., Hilbert, D. M., Rudikoff, S., Ward, J. M., Gonzalez, F. J. Aryl-hydrocarbon receptor-deficient mice are resistant to 2,3,7,8-tetrachlorodibenzo-p-dioxin-induced toxicity. *Toxicol. Appl. Pharmacol.* 1996; 140: 173-179.

Fincham, A. G., Moradian-Oldak, J., Simmer, J. P. The structural biology of the developing dental enamel matrix. *J. Struct. Biol.* 1999; 126: 270-299.

Fincham, A. G., Moradian-Oldak, J., Simmer, J. P., Sarte, P., Lau, E. C., Diekwisch, T., Slavkin, H. C. Self-assembly of a recombinant amelogenin protein generates supramolecular structures. *J. Struct. Biol.* 1994; 112: 103-109.

Fisher, L. W., Torchia, D. A., Fohr, B., Young, M. F., Fedarko, N. S. Flexible structures of SIBLING proteins, bone sialoprotein, and osteopontin. *Biochem. Biophys. Res. Commun.* 2001; 280: 460-465.

Freden, H. and Gronvik, M. Prenatal urinary infection and materialisation of permanent teeth. *Tandlakartidningen* 1980; 72: 1382-1383.

Fujisawa, R., Butler, W. T., Brunn, J. C., Zhou, H. Y., Kuboki, Y. Differences in composition of cell-attachment sialoproteins between dentin and bone. *J. Dent. Res.* 1993; 72: 1222-1226.

Fujiwara, N., Akimoto, T., Otsu, K., Kagiya, T., Ishizeki, K., Harada, H. Reduction of Egf signaling decides transition from crown to root in the development of mouse molars. *J. Exp. Zool. B Mol. Dev. Evol.* 2009; 312B: 486-494.

Fukumoto, S., Kiba, T., Hall, B., Iehara, N., Nakamura, T., Longenecker, G., Krebsbach, P. H., Nanci, A., Kulkarni, A. B., Yamada, Y. Ameloblastin is a cell adhesion molecule required for maintaining the differentiation state of ameloblasts. *J. Cell Biol.* 2004; 167: 973-983.

Gajjeraman, S., Narayanan, K., Hao, J., Qin, C., George, A. Matrix macromolecules in hard tissues control the nucleation and hierarchical assembly of hydroxyapatite. *J. Biol. Chem.* 2007; 282: 1193-1204.

Gao, Y., Sahlberg, C., Kiukkonen, A., Alaluusua, S., Pohjanvirta, R., Tuomisto, J., Lukinmaa, P. L. Lactational exposure of Han/Wistar rats to 2,3,7,8-tetrachlorodibenzo-p-dioxin interferes with enamel maturation and retards dentin mineralization. *J. Dent. Res.* 2004; 83: 139-144.

Gibson, C. W., Yuan, Z. A., Hall, B., Longenecker, G., Chen, E., Thyagarajan, T., Sreenath, T., Wright, J. T., Decker, S., Piddington, R., Harrison, G., Kulkarni, A. B. Amelogenin-deficient mice display an amelogenesis imperfecta phenotype. *J. Biol. Chem.* 2001; 276: 31871-31875.

Goldberg, M., Septier, D., Rapoport, O., Iozzo, R. V., Young, M. F., Ameye, L. G. Targeted disruption of two small leucine-rich proteoglycans, biglycan and decorin, excerpts divergent effects on enamel and dentin formation. *Calcif. Tissue Int.* 2005; 77: 297-310.

Goldberg, M., Septier, D., Rapoport, O., Young, M., Ameye, L. Biglycan is a repressor of amelogenin expression and enamel formation: An emerging hypothesis. *J. Dent. Res.* 2002; 81: 520-524.

Gonzales, F. J. The use of gene knockout mice to unravel the mechanisms of toxicity and chemical carcinogenesis. *Toxicol. Lett.* 2001; 120: 199-208.

Gonzalez, F. J. and Fernandez-Salguero, P. The aryl hydrocarbon receptor: Studies using the AHR-null mice. *Drug Metab. Dispos.* 1998; 26: 1194-1198.

Gorter de Vries, I., Quartier, E., Boute, P., Wisse, E., Coomans, D. Immunocytochemical localization of osteocalcin in developing rat teeth. *J. Dent. Res.* 1987; 66: 784-790.

Gowen, L. C., Petersen, D. N., Mansolf, A. L., Qi, H., Stock, J. L., Tkalcevic, G. T., Simmons, H. A., Crawford, D. T., Chidsey-Frink, K. L., Ke, H. Z., McNeish, J. D., Brown, T. A. Targeted disruption of the osteoblast/osteocyte factor 45 gene (OF45) results in increased bone formation and bone mass. *J. Biol. Chem.* 2003; 278: 1998-2007.

Grace, C. T., Ng, S. K., Cheong, L. L. Recurrent irritant contact dermatitis due to tributyltin oxide on work clothes. *Contact Dermatitis* 1991; 25: 250-251.

Harino, H., Fukushima, M., Kawai, S. Accumulation of butyltin and phenyltin compounds in various fish species. *Arch. Environ. Contam. Toxicol.* 2000; 39: 13-19.

Harmey, D., Hessle, L., Narisawa, S., Johnson, K. A., Terkeltaub, R., Millan, J. L. Concerted regulation of inorganic pyrophosphate and osteopontin by akp2, enpp1, and ank: An integrated model of the pathogenesis of mineralization disorders. *Am. J. Pathol.* 2004; 164: 1199-1209.

Hart, P. S., Hart, T. C., Michalec, M. D., Ryu, O. H., Simmons, D., Hong, S., Wright, J. T. Mutation in kallikrein 4 causes autosomal recessive hypomaturation amelogenesis imperfecta. *J. Med. Genet.* 2004; 41: 545-549.

Hart, T. C. and Hart, P. S. Genetic studies of craniofacial anomalies: Clinical implications and applications. *Orthod. Craniofac. Res.* 2009; 12: 212-220.

Haruyama, N., Sreenath, T. L., Suzuki, S., Yao, X., Wang, Z., Wang, Y., Honeycutt, C., Iozzo, R. V., Young, M. F., Kulkarni, A. B. Genetic evidence for key roles of decorin and biglycan in dentin mineralization. *Matrix Biol.* 2009; 28: 129-136.

Hauschka, P. V., Lian, J. B., Cole, D. E., Gundberg, C. M. Osteocalcin and matrix gla protein: Vitamin K-dependent proteins in bone. *Physiol. Rev.* 1989; 69: 990-1047.

Hayashibara, T., Hiraga, T., Yi, B., Nomizu, M., Kumagai, Y., Nishimura, R., Yoneda, T. A synthetic peptide fragment of human MEPE stimulates new bone formation in vitro and in vivo. *J. Bone Miner. Res.* 2004; 19: 455-462.

He, G., Dahl, T., Veis, A., George, A. Dentin matrix protein 1 initiates hydroxyapatite formation in vitro. *Connect. Tissue Res.* 2003; 44 Suppl 1: 240-245.

He, P., Zhang, Y., Kim, S. O., Radlanski, R. J., Butcher, K., Schneider, R. A., DenBesten, P. K. Ameloblast differentiation in the human developing tooth: Effects of extracellular matrices. *Matrix Biol.* 2010; 29: 411-419.

Heidrich, D. D., Steckelbroeck, S., Klingmuller, D. Inhibition of human cytochrome P450 aromatase activity by butyltins. *Steroids* 2001; 66: 763-769.

Heijs, S. C., Dietz, W., Noren, J. G., Blanksma, N. G., Jalevik, B. Morphology and chemical composition of dentin in permanent first molars with the diagnose MIH. *Swed. Dent. J.* 2007; 31: 155-164.

Heikinheimo, K., Voutilainen, R., Happonen, R. P., Miettinen, P. J. EGF receptor and its ligands, EGF and TGF-alpha, in developing and neoplastic human odontogenic tissues. *Int. J. Dev. Biol.* 1993; 37: 387-396.

Heikkinen, T., Alvesalo, L., Osborne, R. H. Intercuspal distances of the first permanent molar with special reference to maternal smoking during pregnancy. *Acta Med. Auxol.* 1994a; 26: 103-114.

Heikkinen, T., Alvesalo, L., Osborne, R. H., Pirttiniemi, P. Maternal smoking and tooth formation in the foetus. I. tooth crown size in the deciduous dentition. *Early Hum. Dev.* 1992; 30: 49-59.

Heikkinen, T., Alvesalo, L., Osborne, R. H., Sarpola, A. Clinical eruption of permanent incisors and first molars after maternal smoking in pregnancy. *Acta Med. Auxol.* 1995; 27: 83-96.

Heikkinen, T., Alvesalo, L., Osborne, R. H., Tienari, J. Maternal smoking and tooth formation in the foetus. III. thin mandibular incisors and delayed motor development at 1 year of age. *Early Hum. Dev.* 1997; 47: 327-340.

Heikkinen, T., Alvesalo, L., Osborne, R. H., Tienari, J. Maternal smoking and tooth formation in the foetus. II. tooth crown size in the permanent dentition. *Early Hum. Dev.* 1994b; 40: 73-86.

Hoffmann, D. and Hecht, S. S. Advances in tobacco carcinogenesis. In *Chemical Carcinogenesis and Mutagenesis I* (C. S. Cooper and P. L. Grover, Eds.). 1990; 63-102. Springer-Verlag, New Youk.

Hoffmann, D., Hoffmann, I., El-Bayoumy, K. The less harmful cigarette: A controversial issue. a tribute to ernst L. wynder. *Chem. Res. Toxicol.* 2001; 14: 767-790.

Horiguchi, T., Hyeon-Seo, C., Shiraishi, H., Shibata, Y., Soma, M., Morita, M., Shimizu, M. Field studies on imposex and organotin accumulation in the rock shell, thais clavigera, from the seto inland sea and the sanriku region, japan. *Sci. Total Environ.* 1998; 214: 65-70.

Hoshi, K., Amizuka, N., Oda, K., Ikehara, Y., Ozawa, H. Immunolocalization of tissue non-specific alkaline phosphatase in mice. *Histochem. Cell Biol.* 1997; 107: 183-191.

Hoshi, K., Kemmotsu, S., Takeuchi, Y., Amizuka, N., Ozawa, H. The primary calcification in bones follows removal of decorin and fusion of collagen fibrils. *J. Bone Miner. Res.* 1999; 14: 273-280.

Hotton, D., Mauro, N., Lezot, F., Forest, N., Berdal, A. Differential expression and activity of tissue-nonspecific alkaline phosphatase (TNAP) in rat odontogenic cells in vivo. *J. Histochem. Cytochem.* 1999; 47: 1541-1552.

Hu, C. C., Fukae, M., Uchida, T., Qian, Q., Zhang, C. H., Ryu, O. H., Tanabe, T., Yamakoshi, Y., Murakami, C., Dohi, N., Shimizu, M., Simmer, J. P. Cloning and characterization of porcine enamelin mRNAs. *J. Dent. Res.* 1997; 76: 1720-1729.

Hu, J. C., Hu, Y., Smith, C. E., McKee, M. D., Wright, J. T., Yamakoshi, Y., Papagerakis, P., Hunter, G. K., Feng, J. Q., Yamakoshi, F., Simmer, J. P. Enamel defects and ameloblast-specific expression in enam knock-out/lacz knock-in mice. *J. Biol. Chem.* 2008; 283: 10858-10871.

Hu, J. C., Sun, X., Zhang, C., Liu, S., Bartlett, J. D., Simmer, J. P. Enamelysin and kallikrein-4 mRNA expression in developing mouse molars. *Eur. J. Oral Sci.* 2002; 110: 307-315.

Hu, J. C. and Yamakoshi, Y. Enamelin and autosomal-dominant amelogenesis imperfecta. *Crit. Rev. Oral Biol. Med.* 2003; 14: 387-398.

Huang, B., Sun, Y., Maciejewska, I., Qin, D., Peng, T., McIntyre, B., Wygant, J., Butler, W. T., Qin, C. Distribution of SIBLING proteins in the organic and inorganic phases of rat dentin and bone. *Eur. J. Oral Sci.* 2008; 116: 104-112.

Hunter, G. K. and Goldberg, H. A. Nucleation of hydroxyapatite by bone sialoprotein. *Proc. Natl. Acad. Sci. U.S.A.* 1993; 90: 8562-8565.

Hunter, G. K., Hauschka, P. V., Poole, A. R., Rosenberg, L. C., Goldberg, H. A. Nucleation and inhibition of hydroxyapatite formation by mineralized tissue proteins. *Biochem. J.* 1996; 317 (Pt 1): 59-64.

Imai, H., Osumi-Yamashita, N., Ninomiya, Y., Eto, K. Contribution of early-emigrating midbrain crest cells to the dental mesenchyme of mandibular molar teeth in rat embryos. *Dev. Biol.* 1996; 176: 151-165.

Iwata, T., Yamakoshi, Y., Hu, J. C., Ishikawa, I., Bartlett, J. D., Krebsbach, P. H., Simmer, J. P. Processing of ameloblastin by MMP-20. *J. Dent. Res.* 2007; 86: 153-157.

Jalevik, B., Noren, J. G., Klingberg, G., Barregard, L. Etiologic factors influencing the prevalence of demarcated opacities in permanent first molars in a group of swedish children. *Eur. J. Oral Sci.* 2001; 109: 230-234.

Jan, J., Sovcikova, E., Kocan, A., Wsolova, L., Trnovec, T. Developmental dental defects in children exposed to PCBs in eastern slovakia. *Chemosphere* 2007; 67: S350-4.

Jan, J. and Vrbic, V. Polychlorinated biphenyls cause developmental enamel defects in children. *Caries Res.* 2000; 34: 469-473.

Jernvall, J., Aberg, T., Kettunen, P., Keranen, S., Thesleff, I. The life history of an embryonic signaling center: BMP-4 induces p21 and is associated with apoptosis in the mouse tooth enamel knot. *Development* 1998; 125: 161-169.

Jernvall, J., Kettunen, P., Karavanova, I., Martin, L. B., Thesleff, I. Evidence for the role of the enamel knot as a control center in mammalian tooth cusp formation: Non-dividing cells express growth stimulating fgf-4 gene. *Int. J. Dev. Biol.* 1994; 38: 463-469.

Jernvall, J. and Thesleff, I. Reiterative signaling and patterning during mammalian tooth morphogenesis. *Mech. Dev.* 2000; 92: 19-29.

Kaminsky, L. S., Mahoney, M. C., Leach, J., Melius, J., Miller, M. J. Fluoride: Benefits and risks of exposure. *Crit. Rev. Oral Biol. Med.* 1990; 1: 261-281.

Kattainen, H., Tuukkanen, J., Simanainen, U., Tuomisto, J. T., Kovero, O., Lukinmaa, P. L., Alaluusua, S., Tuomisto, J., Viluksela, M. In utero/lactational 2,3,7,8-tetrachlorodibenzo-p-dioxin exposure impairs molar tooth development in rats. *Toxicol. Appl. Pharmacol.* 2001; 174: 216-224.

Kawasaki, K. and Weiss, K. M. Mineralized tissue and vertebrate evolution: The secretory calcium-binding phosphoprotein gene cluster. *Proc. Natl. Acad. Sci. U. S. A.* 2003; 100: 4060-4065.

Keithly, J. C., Cardwell, R. D., Henderson, D. G. Tributyltin in seafood from asia, australia, europe, and north america: Assessment of human health risks. *Hum. Ecol. Risk Assess.* 1999; 5: 337-354.

Khan, M. A., Provenza, D. V., Seibel, W. The effects of nicotine on mouse first molar tooth germs in organ culture. *J. Biol. Buccale* 1981; 9: 335-348.

Kieser, J. A., Groeneveld, H. T., da Silva, P. Delayed tooth formation in children exposed to tobacco smoke. *J. Clin. Pediatr. Dent.* 1996; 20: 97-100.

Kim, J. W., Seymen, F., Lin, B. P., Kiziltan, B., Gencay, K., Simmer, J. P., Hu, J. C. ENAM mutations in autosomal-dominant amelogenesis imperfecta. *J. Dent. Res.* 2005; 84: 278-282.

Kimura, K., Kobayashi, K., Naito, H., Suzuki, Y., Sugita-Konishi, Y. Effect of lactational exposure to tributyltin chloride on innate immunodefenses in the F1 generation in mice. *Biosci. Biotechnol. Biochem.* 2005; 69: 1104-1110.

Kishta, O., Adeeko, A., Li, D., Luu, T., Brawer, J. R., Morales, C., Hermo, L., Robaire, B., Hales, B. F., Barthelemy, J., Cyr, D. G., Trasler, J. M. In utero exposure to tributyltin chloride differentially alters male and female fetal gonad morphology and gene expression profiles in the sprague-dawley rat. *Reprod. Toxicol.* 2007; 23: 1-11.

Kiukkonen, A., Sahlberg, C., Lukinmaa, P. L., Alaluusua, S., Peltonen, E., Partanen, A. M. 2,3,7,8-tetrachlorodibenzo-p-dioxin specifically reduces mRNA for the mineralization-related dentin sialophosphoprotein in cultured mouse embryonic molar teeth. *Toxicol. Appl. Pharmacol.* 2006; 216: 399-406.

Kiukkonen, A., Viluksela, M., Sahlberg, C., Alaluusua, S., Tuomisto, J. T., Tuomisto, J., Lukinmaa, P. L. Response of the incisor tooth to 2,3,7,8-tetrachlorodibenzo-p-dioxin in a dioxin-resistant and a dioxin-sensitive rat strain. *Toxicol. Sci.* 2002; 69: 482-489.

Kiviranta, H., Hallikainen, A., Ovaskainen, M. L., Kumpulainen, J., Vartiainen, T. Dietary intakes of polychlorinated dibenzo-p-dioxins, dibenzofurans and polychlorinated biphenyls in finland. *Food Addit. Contam.* 2001; 18: 945-953.

Kiviranta, H. Exposure and Human PCDD/F and PCB Body Burden in Finland. Publications of the National Public Health Institute KTL A14/2005. Epub: http://www.ktl.fi/portal/suomi/julkaisut/julkaisusarjat/kansanterveyslaitoksen_julkaisuja_a/

Krebsbach, P. H., Lee, S. K., Matsuki, Y., Kozak, C. A., Yamada, K. M., Yamada, Y. Full-length sequence, localization, and chromosomal mapping of ameloblastin. A novel tooth-specific gene. *J. Biol. Chem.* 1996; 271: 4431-4435.

Krishnamachari, K. A. Skeletal fluorosis in humans: A review of recent progress in the understanding of the disease. *Prog. Food Nutr. Sci.* 1986; 10: 279-314.

Laisi, S., Ess, A., Sahlberg, C., Arvio, P., Lukinmaa, P. L., Alaluusua, S. Amoxicillin may cause molar incisor hypomineralization. *J. Dent. Res.* 2009; 88: 132-136.

Laisi, S., Kiviranta, H., Lukinmaa, P. L., Vartiainen, T., Alaluusua, S. Molar-incisor-hypomineralisation and dioxins: New findings. *Eur. Arch. Paediatr. Dent.* 2008; 9: 224-227.

Lézot, F., Davideau, J. L., Thomas, B., Sharpe, P., Forest, N., Berdal, A. Epithelial dlx-2 homeogene expression and cementogenesis. *J. Histochem. Cytochem.* 2000; 48: 277-284.

Lian, J., Stewart, C., Puchacz, E., Mackowiak, S., Shalhoub, V., Collart, D., Zambetti, G., Stein, G. Structure of the rat osteocalcin gene and regulation of vitamin D-dependent expression. *Proc. Natl. Acad. Sci. U. S. A.* 1989; 86: 1143-1147.

Linde, A. and Lundgren, T. From serum to the mineral phase. the role of the odontoblast in calcium transport and mineral formation. *Int. J. Dev. Biol.* 1995; 39: 213-222.

Lu, Y., Ye, L., Yu, S., Zhang, S., Xie, Y., McKee, M. D., Li, Y. C., Kong, J., Eick, J. D., Dallas, S. L., Feng, J. Q. Rescue of odontogenesis in Dmp1-deficient mice by targeted re-expression of DMP1 reveals roles for DMP1 in early odontogenesis and dentin apposition in vivo. *Dev. Biol.* 2007; 303: 191-201.

Lukinmaa, P. L., Sahlberg, C., Leppaniemi, A., Partanen, A. M., Kovero, O., Pohjanvirta, R., Tuomisto, J., Alaluusua, S. Arrest of rat molar tooth development by lactational exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Toxicol. Appl. Pharmacol.* 2001; 173: 38-47.

Lygidakis, N. A., Dimou, G., Marinou, D. Molar-incisor-hypomineralisation (MIH). A retrospective clinical study in greek children. II. possible medical aetiological factors. *Eur. Arch. Paediatr. Dent.* 2008; 9: 207-217.

Mann, K. K., Matulka, R. A., Hahn, M. E., Trombino, A. F., Lawrence, B. P., Kerkvliet, N. I., Sherr, D. H. The role of polycyclic aromatic hydrocarbon metabolism in dimethylbenz[a]anthracene-induced pre-B lymphocyte apoptosis. *Toxicol. Appl. Pharmacol.* 1999; 161: 10-22.

Marlowe, J. L. and Puga, A. Aryl hydrocarbon receptor, cell cycle regulation, toxicity, and tumorigenesis. *J. Cell. Biochem.* 2005; 96: 1174-1184.

Mascarenhas, A. K. Risk factors for dental fluorosis: A review of the recent literature. *Pediatr. Dent.* 2000; 22: 269-277.

Masters, P. M. In vivo decomposition of phosphoserine and serine in noncollagenous protein from human dentin. *Calcif. Tissue Int.* 1985; 37: 236-241.

Matikainen, T., Perez, G. I., Jurisicova, A., Pru, J. K., Schlezinger, J. J., Ryu, H. Y., Laine, J., Sakai, T., Korsmeyer, S. J., Casper, R. F., Sherr, D. H., Tilly, J. L. Aromatic hydrocarbon receptor-driven bax gene expression is required for premature ovarian failure caused by biohazardous environmental chemicals. *Nat. Genet.* 2001; 28: 355-360.

Matikainen, T. M., Moriyama, T., Morita, Y., Perez, G. I., Korsmeyer, S. J., Sherr, D. H., Tilly, J. L. Ligand activation of the aromatic hydrocarbon receptor transcription factor drives bax-dependent apoptosis in developing fetal ovarian germ cells. *Endocrinology* 2002; 143: 615-620.

Matsumoto, Y., Otsuka, F., Takano, M., Mukai, T., Yamanaka, R., Takeda, M., Miyoshi, T., Inagaki, K., Sada, K. E., Makino, H. Estrogen and glucocorticoid regulate osteoblast differentiation through the interaction of bone morphogenetic protein-2 and tumor necrosis factor-alpha in C2C12 cells. *Mol. Cell. Endocrinol.* 2010; 325: 118-127.

May, G. Tetrachlorodibenzodioxin: A survey of subjects ten years after exposure. *Br. J. Ind. Med.* 1982; 39: 128-135.

McKnight, D. A., Suzanne Hart, P., Hart, T. C., Hartsfield, J. K., Wilson, A., Wright, J. T., Fisher, L. W. A comprehensive analysis of normal variation and disease-causing mutations in the human DSPP gene. *Hum. Mutat.* 2008; 29: 1392-1404.

Menzie, C. A., Potocki, B. B., Santodonato, J. Exposure to carcinogenic PAHs in the environment. *Environ. Sci. Technol.* 1992; 26: 1278-1284.

Michon, F., Tummers, M., Kyyronen, M., Frilander, M. J., Thesleff, I. Tooth morphogenesis and ameloblast differentiation are regulated by micro-RNAs. *Dev. Biol.* 2010; 340: 355-368.

Miettinen, H. M., Alaluusua, S., Tuomisto, J., Viluksela, M. Effect of in utero and lactational 2,3,7,8-tetrachlorodibenzo-p-dioxin exposure on rat molar development: The role of exposure time. *Toxicol. Appl. Pharmacol.* 2002; 184: 57-66.

Milan, A. M., Sugars, R. V., Embery, G., Waddington, R. J. Dentinal proteoglycans demonstrate an increasing order of affinity for hydroxyapatite crystals during the transition of predentine to dentine. *Calcif. Tissue Int.* 2004; 75: 197-204.

Mocarelli, P., Marocchi, A., Brambilla, P., Gerthoux, P., Young, D. S., Mantel, N. Clinical laboratory manifestations of exposure to dioxin in children. A six-year study of the effects of an environmental disaster near seveso, italy. *JAMA* 1986; 256: 2687-2695.

Moffatt, P., Smith, C. E., St-Arnaud, R., Simmons, D., Wright, J. T., Nanci, A. Cloning of rat amelotin and localization of the protein to the basal lamina of maturation stage ameloblasts and junctional epithelium. *Biochem. J.* 2006; 399: 37-46.

Moorrees, C. F., Fanning, E. A., Hunt, E. E.,Jr. Age variation of formation stages for ten permanent teeth. *J. Dent. Res.* 1963; 42: 1490-1502.

Moses, M., Lilis, R., Crow, K. D., Thornton, J., Fischbein, A., Anderson, H. A., Selikoff, I. J. Health status of workers with past exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin in the manufacture of 2,4,5-trichlorophenoxyacetic acid: Comparison of findings with and without chloracne. *Am. J. Ind. Med.* 1984; 5: 161-182.

Murshed, M., Schinke, T., McKee, M. D., Karsenty, G. Extracellular matrix mineralization is regulated locally; different roles of two gla-containing proteins. *J. Cell Biol.* 2004; 165: 625-630.

Murtomaa, M., Tervaniemi, O. M., Parviainen, J., Ruokojarvi, P., Tuukkanen, J., Viluksela, M. Dioxin exposure in contaminated sawmill area: The use of molar teeth and bone of bank vole (clethrionomys glareolus) and field vole (microtus agrestis) as biomarkers. *Chemosphere* 2007; 68: 951-957.

Nagano, T., Oida, S., Ando, H., Gomi, K., Arai, T., Fukae, M. Relative levels of mRNA encoding enamel proteins in enamel organ epithelia and odontoblasts. *J. Dent. Res.* 2003; 82: 982-986.

Nampei, A., Hashimoto, J., Hayashida, K., Tsuboi, H., Shi, K., Tsuji, I., Miyashita, H., Yamada, T., Matsukawa, N., Matsumoto, M., Morimoto, S., Ogihara, T., Ochi, T., Yoshikawa, H. Matrix extracellular phosphoglycoprotein (MEPE) is highly expressed in osteocytes in human bone. *J. Bone Miner. Metab.* 2004; 22: 176-184.

Nanci, A. and Bosshardt, D. D. Structure of periodontal tissues in health and disease. *Periodontol. 2000* 2006; 40: 11-28.

Narayanan, K., Ramachandran, A., Hao, J., He, G., Park, K. W., Cho, M., George, A. Dual functional roles of dentin matrix protein 1. implications in biomineralization and gene transcription by activation of intracellular Ca2+ store. *J. Biol. Chem.* 2003; 278: 17500-17508.

Narayanan, K., Srinivas, R., Ramachandran, A., Hao, J., Quinn, B., George, A. Differentiation of embryonic mesenchymal cells to odontoblast-like cells by overexpression of dentin matrix protein 1. *Proc. Natl. Acad. Sci. U. S. A.* 2001; 98: 4516-4521.

Nebert, D. W., Roe, A. L., Dieter, M. Z., Solis, W. A., Yang, Y., Dalton, T. P. Role of the aromatic hydrocarbon receptor and [ah] gene battery in the oxidative stress response, cell cycle control, and apoptosis. *Biochem. Pharmacol.* 2000; 59: 65-85.

Nieminen, P., Papagiannoulis-Lascarides, L., Waltimo-Siren, J., Ollila, P., Karjalainen, S., Arte, S., Veerkamp, J., Walton, V. T., Kustner, E. C., Siltanen, T., Holappa, H., Lukinmaa, P. L., Alaluusua, S. Frameshift mutations in dentin phosphoprotein and dependence of dentin disease phenotype on mutation location. *J. Bone Miner. Res.* 2010;

Noland, E. A., McCauley, P. T., Bull, R. J. Dimethyltin dichloride: Investigations into its gastrointestinal absorption and transplacental transfer. *J. Toxicol. Environ. Health* 1983; 12: 89-98.

Nunez, J., Sanz, M., Hoz-Rodriguez, L., Zeichner-David, M., Arzate, H. Human cementoblasts express enamel-associated molecules in vitro and in vivo. *J. Periodontal. Res.* 2010;

Ogawa, T., Onishi, T., Hayashibara, T., Sakashita, S., Okawa, R., Ooshima, T. Dentinal defects in hyp mice not caused by hypophosphatemia alone. *Arch. Oral Biol.* 2006; 51: 58-63.

Okazaki, R., Inoue, D., Shibata, M., Saika, M., Kido, S., Ooka, H., Tomiyama, H., Sakamoto, Y., Matsumoto, T. Estrogen promotes early osteoblast differentiation and inhibits adipocyte differentiation in mouse bone marrow stromal cell lines that express estrogen receptor (ER) alpha or beta. *Endocrinology* 2002; 143: 2349-2356.

Onishi, T., Ogawa, T., Hayashibara, T., Hoshino, T., Okawa, R., Ooshima, T. Hyper-expression of osteocalcin mRNA in odontoblasts of hyp mice. *J. Dent. Res.* 2005; 84: 84-88.

Ortiz-Delgado, J. B., Simes, D. C., Gavaia, P., Sarasquete, C., Cancela, M. L. Osteocalcin and matrix GLA protein in developing teleost teeth: Identification of sites of mRNA and protein accumulation at single cell resolution. *Histochem. Cell Biol.* 2005; 124: 123-130.

Osuji, O. O., Leake, J. L., Chipman, M. L., Nikiforuk, G., Locker, D., Levine, N. Risk factors for dental fluorosis in a fluoridated community. *J. Dent. Res.* 1988; 67: 1488-1492.

Papagerakis, P., Berdal, A., Mesbah, M., Peuchmaur, M., Malaval, L., Nydegger, J., Simmer, J., Macdougall, M. Investigation of osteocalcin, osteonectin, and dentin sialophosphoprotein in developing human teeth. *Bone* 2002a; 30: 377-385.

Papagerakis, P., MacDougall, M., Berdal, A. Differential epithelial and mesenchymal regulation of tooth-specific matrix proteins expression by 1,25-dihydroxyvitamin D3 in vivo. *Connect. Tissue Res.* 2002b; 43: 372-375.

Papagerakis, P., MacDougall, M., Hotton, D., Bailleul-Forestier, I., Oboeuf, M., Berdal, A. Expression of amelogenin in odontoblasts. *Bone* 2003; 32: 228-240.

Papagerakis, P., Peuchmaur, M., Hotton, D., Ferkdadji, L., Delmas, P., Sasaki, S., Tagaki, T., Berdal, A. Aberrant gene expression in epithelial cells of mixed odontogenic tumors. *J. Dent. Res.* 1999; 78: 20-30.

Partanen, A. M., Alaluusua, S., Miettinen, P. J., Thesleff, I., Tuomisto, J., Pohjanvirta, R., Lukinmaa, P. L. Epidermal growth factor receptor as a mediator of developmental toxicity of dioxin in mouse embryonic teeth. *Lab. Invest.* 1998; 78: 1473-1481.

Partanen, A. M., Kiukkonen, A., Sahlberg, C., Alaluusua, S., Thesleff, I., Pohjanvirta, R., Lukinmaa, P. L. Developmental toxicity of dioxin to mouse embryonic teeth in vitro: Arrest of tooth morphogenesis involves stimulation of apoptotic program in the dental epithelium. *Toxicol. Appl. Pharmacol.* 2004; 194: 24-33.

Partanen, A. M. and Thesleff, I. Localization and quantitation of 125I-epidermal growth factor binding in mouse embryonic tooth and other embryonic tissues at different developmental stages. *Dev. Biol.* 1987; 120: 186-197.

Pirkle, J. L., Wolfe, W. H., Patterson, D. G., Needham, L. L., Michalek, J. E., Miner, J. C., Peterson, M. R., Phillips, D. L. Estimates of the half-life of 2,3,7,8-tetrachlorodibenzo-p-dioxin in vietnam veterans of operation ranch hand. *J. Toxicol. Environ. Health* 1989; 27: 165-171.

Pogany, G., Hernandez, D. J., Vogel, K. G. The in vitro interaction of proteoglycans with type I collagen is modulated by phosphate. *Arch. Biochem. Biophys.* 1994; 313: 102-111.

Pohjanvirta, R. and Tuomisto, J. Short-term toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin in laboratory animals: Effects, mechanisms, and animal models. *Pharmacol. Rev.* 1994; 46: 483-549.

Poser, J. W. and Price, P. A. A method for decarboxylation of gamma-carboxyglutamic acid in proteins. properties of the decarboxylated gamma-carboxyglutamic acid protein from calf bone. *J. Biol. Chem.* 1979; 254: 431-436.

Price, P. A., Parthemore, J. G., Deftos, L. J. New biochemical marker for bone metabolism. measurement by radioimmunoassay of bone GLA protein in the plasma of normal subjects and patients with bone disease. *J. Clin. Invest.* 1980; 66: 878-883.

Price, P. A., Williamson, M. K., Haba, T., Dell, R. B., Jee, W. S. Excessive mineralization with growth plate closure in rats on chronic warfarin treatment. *Proc. Natl. Acad. Sci. U. S. A.* 1982; 79: 7734-7738.

Qin, C., Brunn, J. C., Cadena, E., Ridall, A., Tsujigiwa, H., Nagatsuka, H., Nagai, N., Butler, W. T. The expression of dentin sialophosphoprotein gene in bone. *J. Dent. Res.* 2002; 81: 392-394.

Qin, C., Brunn, J. C., Cook, R. G., Orkiszewski, R. S., Malone, J. P., Veis, A., Butler, W. T. Evidence for the proteolytic processing of dentin matrix protein 1. identification and characterization of processed fragments and cleavage sites. *J. Biol. Chem.* 2003; 278: 34700-34708.

Qin, C., Brunn, J. C., Jones, J., George, A., Ramachandran, A., Gorski, J. P., Butler, W. T. A comparative study of sialic acid-rich proteins in rat bone and dentin. *Eur. J. Oral Sci.* 2001; 109: 133-141.

Qin, C., Huang, B., Wygant, J. N., McIntyre, B. W., McDonald, C. H., Cook, R. G., Butler, W. T. A chondroitin sulfate chain attached to the bone dentin matrix protein 1 NH2-terminal fragment. *J. Biol. Chem.* 2006; 281: 8034-8040.

Rajpar, M. H., Harley, K., Laing, C., Davies, R. M., Dixon, M. J. Mutation of the gene encoding the enamel-specific protein, enamelin, causes autosomal-dominant amelogenesis imperfecta. *Hum. Mol. Genet.* 2001; 10: 1673-1677.

Ramesh, A., Walker, S. A., Hood, D. B., Guillen, M. D., Schneider, K., Weyand, E. H. Bioavailability and risk assessment of orally ingested polycyclic aromatic hydrocarbons. *Int. J. Toxicol.* 2004; 23: 301-333.

Reid, D. J. and Dean, M. C. Variation in modern human enamel formation times. *J. Hum. Evol.* 2006; 50: 329-346.

Risk and Policy Analysts limited (RPA). Risk assessment studies on targeted consumer applications of certain organotin compounds. Final Report prepared for and published by the European Commission. DG Enterprise & Industry. Epub: http://ec.europa.eu/enterprise/chemicals/docs/studies/organotins_3rd_report_16_sept_2005.pdf.

Robinson, C., Connell, S., Kirkham, J., Brookes, S. J., Shore, R. C., Smith, A. M. The effect of fluoride on the developing tooth. *Caries Res.* 2004; 38: 268-276.

Rojas-Sanchez, F., Alaminos, M., Campos, A., Rivera, H., Sanchez-Quevedo, M. C. Dentin in severe fluorosis: A quantitative histochemical study. *J. Dent. Res.* 2007; 86: 857-861.

Romberg, R. W., Werness, P. G., Riggs, B. L., Mann, K. G. Inhibition of hydroxyapatite crystal growth by bone-specific and other calcium-binding proteins. *Biochemistry* 1986; 25: 1176-1180.

Rowe, P. S., Garrett, I. R., Schwarz, P. M., Carnes, D. L., Lafer, E. M., Mundy, G. R., Gutierrez, G. E. Surface plasmon resonance (SPR) confirms that MEPE binds to PHEX via the MEPE-ASARM motif: A model for impaired mineralization in X-linked rickets (HYP). *Bone* 2005; 36: 33-46.

Ruch, J. V., Lesot, H., Begue-Kirn, C. Odontoblast differentiation. *Int. J. Dev. Biol.* 1995; 39: 51-68.

Saad, A. Y., Gartner, L. P., Hiatt, J. L. Teratogenic effects of nicotine on first molar odontogenesis in the mouse. *Acta Morphol. Hung.* 1991; 39: 87-96.

Sahlberg, C., Peltonen, E., Lukinmaa, P. L., Alaluusua, S. Dioxin alters gene expression in mouse embryonic tooth explants. *J. Dent. Res.* 2007; 86: 600-605.

Sahlberg, C., Pohjanvirta, R., Gao, Y., Alaluusua, S., Tuomisto, J., Lukinmaa, P. L. Expression of the mediators of dioxin toxicity, aryl hydrocarbon receptor (AHR) and the AHR nuclear translocator (ARNT), is developmentally regulated in mouse teeth. *Int. J. Dev. Biol.* 2002; 46: 295-300.

Saito, T., Arsenault, A. L., Yamauchi, M., Kuboki, Y., Crenshaw, M. A. Mineral induction by immobilized phosphoproteins. *Bone* 1997; 21: 305-311.

Sarpa, M., De-Carvalho, R. R., Delgado, I. F., Paumgartten, F. J. Developmental toxicity of triphenyltin hydroxide in mice. *Regul. Toxicol. Pharmacol.* 2007; 49: 43-52.

Schonherr, E., Hausser, H., Beavan, L., Kresse, H. Decorin-type I collagen interaction. presence of separate core protein-binding domains. *J. Biol. Chem.* 1995a; 270: 8877-8883.

Schonherr, E., Witsch-Prehm, P., Harrach, B., Robenek, H., Rauterberg, J., Kresse, H. Interaction of biglycan with type I collagen. *J. Biol. Chem.* 1995b; 270: 2776-2783.

Seow, W. K., Romaniuk, K., Sclavos, S. Micromorphologic features of dentin in vitamin D-resistant rickets: Correlation with clinical grading of severity. *Pediatr. Dent.* 1989; 11: 203-208.

Shields, E. D., Bixler, D., el-Kafrawy, A. M. A proposed classification for heritable human dentine defects with a description of a new entity. *Arch. Oral Biol.* 1973; 18: 543-553.

Shimada, T., Sugie, A., Shindo, M., Nakajima, T., Azuma, E., Hashimoto, M., Inoue, K. Tissue-specific induction of cytochromes P450 1A1 and 1B1 by polycyclic aromatic hydrocarbons and polychlorinated biphenyls in engineered C57BL/6J mice of arylhydrocarbon receptor gene. *Toxicol. Appl. Pharmacol.* 2003; 187: 1-10.

Shimasaki, Y., Kitano, T., Oshima, Y., Inoue, S., Imada, N., Honjo, T. Tributyltin causes masculinization in fish. *Environ. Toxicol. Chem.* 2003; 22: 141-144.

Simmer, J. P., Hu, C. C., Lau, E. C., Sarte, P., Slavkin, H. C., Fincham, A. G. Alternative splicing of the mouse amelogenin primary RNA transcript. *Calcif. Tissue Int.* 1994; 55: 302-310.

Simmer, J. P., Hu, Y., Lertlam, R., Yamakoshi, Y., Hu, J. C. Hypomaturation enamel defects in Klk4 knockout/LacZ knockin mice. *J. Biol. Chem.* 2009; 284: 19110-19121.

Singh, S. K., Pal Bhadra, M., Girschick, H. J., Bhadra, U. MicroRNAs--micro in size but macro in function. *FEBS J.* 2008; 275: 4929-4944.

Smith, B. S. Tributyltin compounds induce male characteristics on female mud snails nassarius obsoletus = ilyanassa obsoleta. *J. Appl. Toxicol.* 1981; 1: 141-144.

Smith, C. E. Cellular and chemical events during enamel maturation. *Crit. Rev. Oral Biol. Med.* 1998; 9: 128-161.

Smith, C. E., Nanci, A., Denbesten, P. K. Effects of chronic fluoride exposure on morphometric parameters defining the stages of amelogenesis and ameloblast modulation in rat incisors. *Anat. Rec.* 1993; 237: 243-258.

Smith, C. E. and Warshawsky, H. Quantitative analysis of cell turnover in the enamel organ of the rat incisor. evidence for ameloblast death immediately after enamel matrix secretion. *Anat. Rec.* 1977; 187: 63-98.

Speirs, R. L. The relationship between fluoride concentrations in serum and in mineralized tissues in the rat. *Arch. Oral Biol.* 1986; 31: 373-381.

Sreenath, T., Thyagarajan, T., Hall, B., Longenecker, G., D'Souza, R., Hong, S., Wright, J. T., MacDougall, M., Sauk, J., Kulkarni, A. B. Dentin sialophosphoprotein knockout mouse teeth display widened predentin zone and

develop defective dentin mineralization similar to human dentinogenesis imperfecta type III. *J. Biol. Chem.* 2003; 278: 24874-24880.

Strand, J. and Jacobsen, J. A. Accumulation and trophic transfer of organotins in a marine food web from the danish coastal waters. *Sci. Total Environ.* 2005; 350: 72-85.

Suga, S. Enamel hypomineralization viewed from the pattern of progressive mineralization of human and monkey developing enamel. *Adv. Dent. Res.* 1989; 3: 188-198.

Suzuki, N., Tabata, M. J., Kambegawa, A., Srivastav, A. K., Shimada, A., Takeda, H., Kobayashi, M., Wada, S., Katsumata, T., Hattori, A. Tributyltin inhibits osteoblastic activity and disrupts calcium metabolism through an increase in plasma calcium and calcitonin levels in teleosts. *Life Sci.* 2006; 78: 2533-2541.

Suzuki, S., Sreenath, T., Haruyama, N., Honeycutt, C., Terse, A., Cho, A., Kohler, T., Muller, R., Goldberg, M., Kulkarni, A. B. Dentin sialoprotein and dentin phosphoprotein have distinct roles in dentin mineralization. *Matrix Biol.* 2009; 28: 221-229.

ten Cate, J. M. Current concepts on the theories of the mechanism of action of fluoride. *Acta Odontol. Scand.* 1999; 57: 325-329.

ten Tusscher, G. W., Stam, G. A., Koppe, J. G. Open chemical combustions resulting in a local increased incidence of orofacial clefts. *Chemosphere* 2000; 40: 1263-1270.

Thesleff, I. The genetic basis of tooth development and dental defects. *Am. J. Med. Genet. A.* 2006; 140: 2530-2535.

Thesleff, I. Epithelial-mesenchymal signalling regulating tooth morphogenesis. *J. Cell. Sci.* 2003; 116: 1647-1648.

Thesleff, I. and Hurmerinta, K. Tissue interactions in tooth development. *Differentiation* 1981; 18: 75-88.

Thomas, H. F. and Kollar, E. J. Differentiation of odontoblasts in grafted recombinants of murine epithelial root sheath and dental mesenchyme. *Arch. Oral Biol.* 1989; 34: 27-35.

Thylstrup, A. and Fejerskov, O. A scanning electron microscopic and microradiographic study of pits in fluorosed human enamel. *Scand. J. Dent. Res.* 1979; 87: 105-114.

Thylstrup, A. and Fejerskov, O. Clinical appearance of dental fluorosis in permanent teeth in relation to histologic changes. *Community Dent. Oral Epidemiol.* 1978; 6: 315-328.

Tindall, J. P. Chloracne and chloracnegens. *J. Am. Acad. Dermatol.* 1985; 13: 539-558.

Tompkins, K. Molecular mechanisms of cytodifferentiation in mammalian tooth development. *Connect. Tissue Res.* 2006; 47: 111-118.

Tompkins, K., Alvares, K., George, A., Veis, A. Two related low molecular mass polypeptide isoforms of amelogenin have distinct activities in mouse tooth germ differentiation in vitro. *J. Bone Miner. Res.* 2005; 20: 341-349.

Tsuchiya, M., Sharma, R., Tye, C. E., Sugiyama, T., Bartlett, J. D. Transforming growth factor-beta1 expression is up-regulated in maturation-stage enamel organ and may induce ameloblast apoptosis. *Eur. J. Oral Sci.* 2009; 117: 105-112.

Tsukamoto, Y., Ishihara, Y., Miyagawa-Tomita, S., Hagiwara, H. Inhibition of ossification in vivo and differentiation of osteoblasts in vitro by tributyltin. *Biochem. Pharmacol.* 2004; 68: 739-746.

Uchida, T., Tanabe, T., Fukae, M., Shimizu, M., Yamada, M., Miake, K., Kobayashi, S. Immunochemical and immunohistochemical studies, using antisera against porcine 25 kDa amelogenin, 89 kDa enamelin and the 13-17 kDa nonamelogenins, on immature enamel of the pig and rat. *Histochemistry* 1991; 96: 129-138.

Uno, S., Dalton, T. P., Derkenne, S., Curran, C. P., Miller, M. L., Shertzer, H. G., Nebert, D. W. Oral exposure to benzo[a]pyrene in the mouse: Detoxication by inducible cytochrome P450 is more important than metabolic activation. *Mol. Pharmacol.* 2004; 65: 1225-1237.

USEPA. U.S. Environmental Protection Agency. In: http://cfpub.epa.gov/ncea/cfm/part1and2.cfm?ActType=default

Vaahtokari, A., Aberg, T., Thesleff, I. Apoptosis in the developing tooth: Association with an embryonic signaling center and suppression by EGF and FGF-4. *Development* 1996; 122: 121-129.

Van den Berg, M., Birnbaum, L. S., Denison, M., De Vito, M., Farland, W., Feeley, M., Fiedler, H., Hakansson, H., Hanberg, A., Haws, L., Rose, M., Safe, S., Schrenk, D., Tohyama, C., Tritscher, A., Tuomisto, J., Tysklind, M., Walker, N., Peterson, R. E. The 2005 World Health Organization reevaluation of human and Mammalian toxic equivalency factors for dioxins and dioxin-like compounds. *Toxicol. Sci.* 2006; 93: 223-241.

Van den Berg, M., De Jongh, J., Poiger, H., Olson, J. R. The toxicokinetics and metabolism of polychlorinated dibenzo-p-dioxins (PCDDs) and dibenzofurans (PCDFs) and their relevance for toxicity. *Crit. Rev. Toxicol.* 1994; 24: 1-74.

van den Bos, T., Handoko, G., Niehof, A., Ryan, L. M., Coburn, S. P., Whyte, M. P., Beertsen, W. Cementum and dentin in hypophosphatasia. *J. Dent. Res.* 2005; 84: 1021-1025.

van Leeuwen, F. X. R. and Malisch, R. Results of the third round of the WHO-coordinated exposure study on the levels of PCBs, PCDDs and PCDFs in human milk. *Organohalogen Compounds* 2002; 56: 311-316.

Vartiainen, T., Saarikoski, S., Jaakkola, J. J., Tuomisto, J. PCDD, PCDF, and PCB concentrations in human milk from two areas in finland. *Chemosphere* 1997; 34: 2571-2583.

Vaux, D. L. and Korsmeyer, S. J. Cell death in development. *Cell* 1999; 96: 245-254.

von Marschall, Z. and Fisher, L. W. Dentin sialophosphoprotein (DSPP) is cleaved into its two natural dentin matrix products by three isoforms of bone morphogenetic protein-1 (BMP1). *Matrix Biol.* 2010; 29: 295-303.

Walton, K., Dorne, J. L., Renwick, A. G. Uncertainty factors for chemical risk assessment: interspecies differences in the in vivo pharmacokinetics and metabolism of human CYP1A2 substrates. *Food Chem. Toxicol.* 2001; 39: 667-680.

Wang, H. G., Kawashima, N., Iwata, T., Xu, J., Takahashi, S., Sugiyama, T., Suda, H. MEPE Activated by Furin Promotes Pulpal Cell Adhesion. *J. Dent. Res.* 2011; 90: 529-534.

Wang, X. P., Suomalainen, M., Jorgez, C. J., Matzuk, M. M., Werner, S., Thesleff, I. Follistatin regulates enamel patterning in mouse incisors by asymmetrically inhibiting BMP signaling and ameloblast differentiation. *Dev. Cell.* 2004; 7: 719-730.

Wax, P. M. and Dockstader, L. Tributyltin use in interior paints: A continuing health hazard. *J. Toxicol. Clin. Toxicol.* 1995; 33: 239-241.

Waymire, K. G., Mahuren, J. D., Jaje, J. M., Guilarte, T. R., Coburn, S. P., MacGregor, G. R. Mice lacking tissue non-specific alkaline phosphatase die from seizures due to defective metabolism of vitamin B-6. *Nat. Genet.* 1995; 11: 45-51.

Weerheijm, K. L. and Mejare, I. Molar incisor hypomineralization: A questionnaire inventory of its occurrence in member countries of the european academy of paediatric dentistry (EAPD). *Int. J. Paediatr. Dent.* 2003; 13: 411-416.

Whitlock, J. P.,Jr. Induction of cytochrome P4501A1. *Annu. Rev. Pharmacol. Toxicol.* 1999; 39: 103-125.

Whyte, M. P. Hypophosphatasia and the role of alkaline phosphatase in skeletal mineralization. *Endocr. Rev.* 1994; 15: 439-461.

Wilhelm, M., Ewers, U., Wittsiepe, J., Furst, P., Holzer, J., Eberwein, G., Angerer, J., Marczynski, B., Ranft, U. Human biomonitoring studies in north rhine-westphalia, germany. *Int. J. Hyg. Environ. Health* 2007; 210: 307-318.

Witkop, C. J.,Jr. Amelogenesis imperfecta, dentinogenesis imperfecta and dentin dysplasia revisited: Problems in classification. *J. Oral Pathol.* 1988; 17: 547-553.

Woltgens, J. H., Lyaruu, D. M., Bervoets, T. J., Bronckers, A. L. Effects of calcium and phosphate on secretion of enamel matrix and its subsequent mineralization in vitro. *Adv. Dent. Res.* 1987; 1: 196-201.

Wright, J. T., Frazier-Bowers, S., Simmons, D., Alexander, K., Crawford, P., Han, S. T., Hart, P. S., Hart, T. C. Phenotypic variation in FAM83H-associated amelogenesis imperfecta. *J. Dent. Res.* 2009a; 88: 356-360.

Wright, J. T., Hart, P. S., Aldred, M. J., Seow, K., Crawford, P. J., Hong, S. P., Gibson, C. W., Hart, T. C. Relationship of phenotype and genotype in X-linked amelogenesis imperfecta. *Connect. Tissue Res.* 2003; 44 Suppl 1: 72-78.

Wright, J. T., Hart, T. C., Hart, P. S., Simmons, D., Suggs, C., Daley, B., Simmer, J., Hu, J., Bartlett, J. D., Li, Y., Yuan, Z. A., Seow, W. K., Gibson, C. W. Human and mouse enamel phenotypes resulting from mutation or altered expression of AMEL, ENAM, MMP20 and KLK4. *Cells Tissues Organs* 2009b; 189: 224-229.

Wright, J. T., Johnson, L. B., Fine, J. D. Development defects of enamel in humans with hereditary epidermolysis bullosa. *Arch. Oral. Biol.* 1993; 38: 945-955.

Wurtz, T., Houari, S., Mauro, N., MacDougall, M., Peters, H., Berdal, A. Fluoride at non-toxic dose affects odontoblast gene expression in vitro. *Toxicology* 2008; 249: 26-34.

Yamakoshi, Y., Hu, J. C., Fukae, M., Zhang, H., Simmer, J. P. Dentin glycoprotein: The protein in the middle of the dentin sialophosphoprotein chimera. *J. Biol. Chem.* 2005; 280: 17472-17479.

Yamakoshi, Y., Hu, J. C., Iwata, T., Kobayashi, K., Fukae, M., Simmer, J. P. Dentin sialophosphoprotein is processed by MMP-2 and MMP-20 in vitro and in vivo. *J. Biol. Chem.* 2006; 281: 38235-38243.

Yang, X., Wang, L., Qin, Y., Sun, Z., Henneman, Z. J., Moradian-Oldak, J., Nancollas, G. H. How amelogenin orchestrates the organization of hierarchical elongated microstructures of apatite. *J. Phys. Chem. B.* 2010; 114: 2293-2300.

Ye, L., MacDougall, M., Zhang, S., Xie, Y., Zhang, J., Li, Z., Lu, Y., Mishina, Y., Feng, J. Q. Deletion of dentin matrix protein-1 leads to a partial failure of maturation of predentin into dentin, hypomineralization, and expanded cavities of pulp and root canal during postnatal tooth development. *J. Biol. Chem.* 2004; 279: 19141-19148.

Yoshida, T., Miyoshi, J., Takai, Y., Thesleff, I. Cooperation of nectin-1 and nectin-3 is required for normal ameloblast function and crown shape development in mouse teeth. *Dev. Dyn.* 2010; 239: 2558-2569.

Zanieri, L., Galvan, P., Checchini, L., Cincinelli, A., Lepri, L., Donzelli, G. P., Del Bubba, M. Polycyclic aromatic hydrocarbons (PAHs) in human milk from italian women: Influence of cigarette smoking and residential area. *Chemosphere* 2007; 67: 1265-1274.

Zeichner-David, M. Regeneration of periodontal tissues: cementogenesis revisited. *Periodontol. 2000.* 2006; 41: 196-217.

Zeichner-David, M., Oishi, K., Su, Z., Zakartchenko, V., Chen, L. S., Arzate, H., Bringas, P.,Jr. Role of hertwig's epithelial root sheath cells in tooth root development. *Dev. Dyn.* 2003; 228: 651-663.

Zhang, B., Sun, Y., Chen, L., Guan, C., Guo, L., Qin, C. Expression and distribution of SIBLING proteins in the predentin/dentin and mandible of hyp mice. *Oral Dis.* 2010; 16: 453-464.

ACKNOWLEDGEMENTS

This thesis work was carried out in the research group of Professor Satu Alaluusua and Docent Pirjo-Liisa Lukinmaa at the Department of Pediatric and Preventive Dentistry, Institute of Dentistry, Faculty of Medicine, University of Helsinki during the years 2002-2011. I thank the Institute of Dentistry and the Dean, Professor Jarkko Hietanen, for providing the facilities for my research. I also want to thank the Helsinki Biomedical Graduate School for selecting me to enter the DDS, PhD Program, and for financial support and organized courses and seminars.

I am deeply grateful to Professor Satu Alaluusua and Docent Pirjo-Liisa Lukinmaa for their supervision and all the support and help, particularly related to finishing my thesis. I thank Satu for the excellent ideas and assistance with the statistics. I thank Pirjo-Liisa for teaching me how to write scientifically and carefully reviewing all of my writing.

I thank my official referees, Professor Irma Thesleff and Docent Matti Viluksela, for their constructive comments and great ideas on how to improve the thesis. I also wish to thank my Thesis Committee members Professor Thesleff and Hannu Kiviranta, PhD, for giving advice and comments on my research over the years.

I thank Doctor Jennifer Rowland for the language editing of my thesis manuscript.

I am very grateful to Carin Sahlberg, MSc, for co-authorship, teaching me everything in the Lab, and always being there ready to solve my technical problems. I also thank Carin for Figure 1.

I wish to warmly thank the members of our research group: Anna-Maija Partanen, PhD, DDS, for co-authorship and a great deal of scientific support and discussion; Anu Kiukkonen, PhD, DDS, for co-authorship, giving me great methodological advice, teaching me how to make images, and particularly for friendship; and Anneli Ess, DDS, for friendship.

I am grateful to Marjatta Kivekäs, Maarit Hakkarainen, Pirjo Jutila and Raila Jalomeri, for their excellent technical help. I also want to thank Docent Pekka Nieminen, for scientific discussion, and all of my colleagues at the Institute of Dentistry.

I am thankful for the financial support that I have received from the Finnish Dental Society Apollonia and the Biomedicum Helsinki Foundation.

My warmest thanks go to Lotta Veistinen, DDS, for friendship, sharing thoughts and listening to my worries. I also want to thank Suvi-Tuuli Vilén, DDS, Lotta Seppänen, DDS, Sofia Oja, BSc, Heidi Holappa, DDS, and Maarit Takatalo, PhD, for friendship and lunchbreak company.

I thank my parents, Ritva and Erkki, for always encouraging me to study; they have nurtured my endless enthusiasm for finding out things about Nature. I also thank my brother, Risto, for being there fore me.

Finally, my sincerest and warmest thanks belong to my beloved husband, Antti, for all the help, encouragement and support. I am particularly grateful for your patience and taking care of our household during these last couple of months when I have been editing my thesis. I thank our dear son, Niilo, for the happy smiles and kisses, and for reminding me about the priorities in my life.